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Role of B-Raf^{V600E} in differentiated thyroid cancer and preclinical validation of compounds against B-Raf^{V600E}

Carmelo Nucera[1], **Melanie Goldfarb**[2], **Richard Hodin**[1], and **Sareh Parangi**[1]

[1]Endocrine Surgery Department, Thyroid Cancer Research Laboratory, Massachusetts General Hospital, Harvard Medical School, Boston, MA, USA

[2]Beth Israel Deaconess Medical Center, and Harvard Medical School, Boston, MA, USA

Summary

B-Raf^{V600E}, an oncogenic protein kinase, is the most frequent genetic alteration in papillary thyroid carcinomas (PTC). PTC represents 80–90% of all thyroid cancers and over the past five years, more than 200 manuscripts have been published about the relationship between "B-Raf^{V600E} and thyroid cancer". B-Raf^{V600E} genetically arises from a transversion point mutation (valine-to-glutamate substitution at amino acid residue-600, V600E) and leads to over activation of the mitogen-activated protein kinases (MAPK) signalling pathway. The MAPK pathway is essential for transmitting proliferation signals generated by cell surface receptors and cytoplasmic signaling elements to the nucleus. In many cancers, including thyroid cancer, B-Raf^{V600E} appears to play a crucial role in cell proliferation, survival and de-differentiation. In thyroid cancer, the V600E mutation occurs with greater frequently in aggressive subtypes of PTC, and in individuals that present at advanced stages of disease with extra-thyroidal extension and/or lymph node metastases. B-Raf^{V600E} is considered a marker of aggressive disease in both PTC (>1 cm) and micro-PTC (≤1 cm), and interestingly, is associated with both loss of I-131 avidity and PTC recurrence. Though treatment of patients with thyroid cancer is usually successful and most patients are rendered disease-free, to date there are no effective therapies for patients with invasive, non-radioiodine sensitive tumors or metastatic disease. In this article we will review the relation between B-Raf^{V600E} and PTC, as well as both non-selective and selective pharmacological agents currently under investigation for treatment of B-Raf^{V600E} positive PTC.

Keywords

differentiated thyroid cancer; papillary thyroid cancer; papillary thyroid microcarcinoma; extrathyroidal extension; wild-type B-Raf; B-Raf^{V600E}; MAP kinases; compounds; preclinical validations

Corresponding author information: Sareh Parangi, MD, Assistant Professor of Surgery, Harvard Medical School, Massachusetts General Hospital, Division of General and Gastrointestinal Surgery, Wang ACC 460, 15 Parkman Street, Boston, MA 02115, sparangi@partners.org, Phone: 617-643-4806, Fax: 617- 643-4802.

1. Introduction

1.1 Incidence and genetic alterations of Differentiated Thyroid Cancer

Differentiated thyroid cancer (DTC), though relatively uncommon, is the most frequent endocrine malignancy. As the cancer with the most rapidly increasing rate among women and the second most rapid among men [1], novel therapeutic options may be needed especially for the most aggressive subtypes. In 2007, approximately 33,000 new cases of DTC were diagnosed in the United States with approximately 28,000 papillary, 3,500 follicular and Hurthle cell, 1,300 medullary, and 600 anaplastic carcinomas. This reflects an increased incidence of almost 240% during the later half of the twentieth century, likely related to the improvements in earlier detection of subclinical disease, such as micro-PTCs.

DTC is currently 2 to 3 times more common in women, more aggressive in men and the prognosis is less favorable in men than in women, and with its incidence increasing by almost 5% each year, it currently ranks as the eighth most common malignancy diagnosed in women, though mortality rates are higher for men [2]. Although thyroid carcinoma can occur at any age, the peak incidence is around age 50 to 54 in women and 65 to 69 in men. It accounts for 7.5% to 10% of all diagnosed malignancies among individuals aged 15 to 24, and is also diagnosed more frequently in white North Americans in comparison to African Americans. DTC includes papillary (PTCs) and follicular (FTCs) subtypes that are characterized by well-defined, though evolving, morphologic criteria. PTC is described histologically as a malignant tumor exhibiting evidence of follicular epithelial differentiation and marked by distinctive nuclear features. PTC accounts for 85–90% of all thyroid malignancies, presents with local lymph node involvement less than 50% of the time and metastasizes to distant foci in 5–7% of cases. While incidence of PTC continues to increase, that of FTC is on the decline, possibly due to the changing recognition of nuclear morphological criteria [3].

Although there are many modalities available for the treatment of differentiated thyroid carcinoma, there has been little progress in improving overall survival for this malignancy. In the United States more than 1,500 people die of thyroid cancer each year, with upwards of 35,000 people worldwide (data from 2002). Over one third of these deaths are attributed to the small percentage of undifferentiated/anaplastic carcinomas. While the typical treatment regimen of surgical excision, oral levothyroxine suppression, and radioactive iodine when needed is successful in close to 90% of patients with DTC, for the group of patients that fail to respond to this treatment paradigm or present initially with aggressive tumors, survival rates are very low and there are few therapeutic options [2]. Traditional cytotoxic chemotherapy protocols have shown response rates of 20% at best, and with little long term efficacy. As a result, the lack of alternative therapies for aggressive or recurrent thyroid cancer has become ever more obvious, spurring the clinical research for novel molecular therapeutic targets into the pharmaceutical spotlight.

Prior to 2002, no clinical trials or novel therapeutics existed for treating DTC. In 2008, a significant number of phase 1 and 2 trials that target genetic alterations such as B-Raf, RET or vascular endothelial growth factor (VEGF) and VEGF receptor are available to patients with aggressive and recurrent thyroid cancer. B-Raf^{V600E} mutation is the most prevalent genetic alterations implicated in the initiation and progression of papillary thyroid cancer, thus inhibiting its pathway is the most promising avenue for developing targeted therapies. Theories of PTC pathogenesis are based on a premise of abnormal over-activation of the Ras/RAF/MAPK (mitogen-activated extracellular signal regulated kinase) signaling pathway due to a RET/PTC translocation (10–50% of PTCs) [4, 5], Ras mutations (about 12% of PTCs) [6], or B-Raf^{V600E} (29–83% of PTCs) [7, 8].

This review will specifically focus on the B-Raf^{V600E} mutated gene, its involvement in the pathogenesis of differentiated thyroid cancer, and the exciting new therapies associated with B-Raf^{V600E} positive PTC.

2. Wild-type B-Raf versus B-Raf^{V600E}

2.1 Raf kinases: a long history

In 1983, the transforming replication-defective mouse type C virus 3611-MSV was cloned and characterized. As a retroviral oncogene that induces the growth of fibrosarcoma in mice, the transduced oncogene was named v-Raf for “rapidly accelerated fibrosarcoma” and its cellular homologue C-Raf [9]. All known mammalian Raf kinase isoforms, A, B and C-Raf (also named Raf-1), are cytosolic protein kinases and play key roles in various physiological processes such as cellular metabolism, cell cycle regulation, cell death, and neurological function [10]. Interestingly, Raf homologues were discovered in various organisms such as invertebrates, birds, bony fish, and amphibians and many of the initial Raf studies were performed in invertebrates. Whereas mammals express three isoforms of Raf, invertebrates have only one Raf isoform resembling the mammalian B-Raf isoform [10]. C-Raf is expressed ubiquitously, whereas B-Raf is expressed at higher levels in a variety of human tissues such as hemopoietic cells, neurons, testis, and including thyroid follicular cells [11]. Importantly, for all isoforms, activation segment phosphorylation and, in the case of A-Raf and C-Raf, N-region phosphorylation occur at the plasma membrane, in part explaining why membrane recruitment is essential for Raf activation by small G-protein Ras and membrane-bound receptors, and this is an essential step for their activation by growth factors, cytokines, and hormones. [12]. However, B-Raf’s basal kinase activity is much higher than both A-Raf and C-Raf; B-Raf is fully activated by Ras alone, whereas A-Raf and C-Raf require both Ras and src-proteins tyrosine kinases for activation [12]. While initially, most research focused on the C-Raf isoform and its role as an immediate downstream target of the Ras proteins, researchers soon realized that in fact, B-Raf was the isoform mutated in most human cancers.

In 2002, Davies et al. identified an oncogene widespread amongst human cancers, mutant V600E B-Raf, or B-Raf^{V600E}. B-Raf^{V600E} is expressed in a variety human cancer cell lines including melanoma, colorectal, glioma, sarcoma, breast and thyroid cancer [13, 14]. For example, the B-Raf^{V600E} allele is found in as many as 80% of benign skin nevi. Though no direct evidence yet exists that benign nevi harboring B-Raf^{V600E} eventually progress to malignancy, many cases likely represent terminally differentiated lesions that happen to contain the B-Raf^{V600E} mutation, which is analogous to non dysplastic colorectal aberrant crypt foci where foci that harbour K-Ras mutations without APC mutations have very low malignant potential. However, the mutant allele may play a role in initial nevus formation and subsequent melanoma initiation and is currently under investigation. Another example is colorectal cancer, where there is a strong association between mutant B-Raf^{V600E} and DNA mismatch repair (MMR) system deficient genes [15]. The presence of B-Raf mutations in a high percentage of colorectal MMR-deficient carcinomas suggests its tumorigenic involvement within these tumors, and also its capability to induce tumor cell clonal expansions. Furthermore, the absence of concomitant K-Ras and B-Raf mutations in these tumors has suggested that both are alternative genetic events in colorectal tumorigenesis, and also that alterations within the Ras/RAF/MAPK pathway characterize MMR-deficient colorectal tumors accordingly [15].

Importantly, though having coincident mutations in B-Raf and Ras is quite rare in human cancer, about 30% of the B-Raf inactive mutants (i.e D594) occur in human cancers that also harbor Ras mutations and could act in a dominant negative manner to suppress excessive Ras-MAP kinase signalling [12]. Therefore, while it is highly suggestive that the Ras–Raf–

MAP kinase pathway plays a dominant role in a wide array of cancer types, teasing out at what level the initial mutation occurs in the pathway for each particular cancer, i.e. whether the B-Raf or Ras specific genetic aberration is the cause of tumorigenesis, is a difficult task. With a seemingly growing importance in tumorigenesis, B-Raf^{V600E} is being closely studied as a potential target for more effective oncologic therapies.

2.2 Wild-type B-Raf

The wild-type B-Raf gene is located on human chromosome 7q24. The gene encodes a cytosolic serine–threonine protein kinase that is expressed in thyroid follicular cells [11]. B-Raf, when compared with the other two functional human Raf proteins A-Raf and C-Raf, has the highest basal kinase activity and is the most potent activator of mitogen-activated extracellular-regulated protein kinase (MPAK) signalling pathway. B-Raf activates MAPKK (MEK) and MEK phosphorylates MAPK (ERK), all of which are required for maintaining vertebrate cellular activities responsive to growth factors stimulation [10, 16]. Both B-Raf and MAP kinases are dependent upon up-stream Ras and tyrosine kinase receptor activation (Figure 1). All together they form a molecular axis crucial in the regulation of gene expression, cell proliferation, and differentiation [17–19].

Similar to all RAF kinases, B-Raf is comprised of three homologous regions, CR1, CR2, and CR3. CR1 has all elements essential to Raf membrane recruitment. These include a Ras binding domain (RBD) that binds to the active Ras-GTP, and a cysteine rich domain (CRD) that stabilizes its interaction with Ras [10]. CR2, the N-terminal regulatory domain, contains a conserved phosphorylation site at S259 that serves as a regulatory binding site for protein 14-3-3, an essential dimeric cofactor for Raf kinase activity [10]. The N-terminal domain also acts as an autoinhibitor of the C-terminal kinase domain and helps to maintain B-Raf in an inactive state in the absence of extracellular stimuli [17]. Lastly, CR3, the C-terminal kinase catalytic domain, serves as the catalytic portion of the B-Raf kinase domain. CR3 is folded and bound to the N-terminal domain in a conformation stabilized by protein 14-3-3 at two different sites, one being a conserved serine at S621 within the CR3 region [10].

B-Raf exhibits a characteristic bilobar structure similar to all protein kinases. When in an inactive conformation, B-Raf residue G596-V600 in the activation loop forms a hydrophobic interaction with residues G464-V471 in the ATP-binding site (P loop), resulting in a structure that does not allow binding to ATP or substrate. Most, but not all of the known oncogenic B-Raf substitutions promote the formation of new interactions, folding the kinase into a catalytically competent structure. Those mutations that occur in the activation loop or the P loop, destabilize the hydrophobic interaction and disrupt the inactive conformation. In contrast, some B-Raf mutants impair in vitro kinase activity, and though these mutants have lower kinase activity, they are able to activate C-Raf and induce ERK phosphorylation, presumably by heterodimerization [20].

B-Raf protein is activated at the membrane through a complex process that involves multiple phosphorylation events and protein/lipid interactions (Figure 1). The inactive conformation of B-Raf involves the simultaneous binding of 14-3-3 to phosphorylated sites S365 and S729. To prime for activation, B-Raf is phosphorylated at site S446, leading to a maximally negatively charged amino-region. Next, extracellular signals (i.e. mitogens, hormones, and neurotransmitters) act on Ras-GTP to induce a tyrosine kinase receptor and initiate the activation sequence. Pre-activated B-Raf is then recruited to the plasma membrane where it is fully activated, first as two protein phosphatases (PP1 and PP2A) dephosphorylate the N-terminal 14-3-3-binding sites, and second by phosphorylation of the B-Raf kinase domain, converting it to its active form [17] (Figure 1). Importantly, oncogenic Ras phosphorylates two conserved sites of B-Raf, T598 and S601. This event not only renders B-Raf

constitutively active, but also induces ERK1/2 activation, causing the transformation of normal cells [21, 22].

2.2 B-Raf^{V600E}

B-Raf^{V600E} is an oncogenic protein with markedly elevated kinase activity that over-activates the MAP kinase pathway [13, 23]. The B-Raf^{V600E} mutation occurs with a frequency of approximately 8% and ranks only behind Ras (15%) as the second most common somatic mutation observed in human cancer. In comparison to wild-type B-Raf, B-Raf^{V600E} transforms normal cells, and does not require Ras function to induce growth of human cancer cells (Figure 2) [13]. The mutation places B-Raf in a constitutively active state by inserting a negatively charged residue adjacent to the phosphorylation site at T598 and mimicking phosphorylation at Thr^{599} and Ser^{602} residues. Thus B-Raf^{V600E} assumes a three-dimensional conformation which corresponds to the activated state of B-Raf.

All B-Raf mutations occur in two distinct regions of the B-Raf kinase domain: the glycine-rich loop and the kinase activation segment. More than 80% of these mutations correspond to a T→A transversion at position 1799 (B-Raf^{V600E}, somatic missense mutation) that results in the substitution of Valine by Glutamate at position 600 (V600E) of the kinase activation segment characterized by four negative charges in the amino-terminal domain that renders B-Raf unique kinase and constitutively active [12, 13, 24].

B-Raf is the only Raf gene mutated with any regularity across the spectrum of human cancers, including melanoma (up to 66%), papillary thyroid carcinoma (between 29% and 83%), ovarian carcinoma (14%), colonic adenocarcinoma (12%), and sarcoma (0.05%) [13, 24]. In PTC and other human cancers, the B-Raf^{V600E} mutation does not overlap with other genetic alterations (i.e. Ras mutations, RET/PTC translocations), suggesting that human differentiated cancer cells may require fewer mutational events to induce cellular transformational change and B-Raf^{V600E} is an integral propagator of tumorigenesis [25]. Conversely, B-Raf^{V600E} mutation might occur concomitantly and work synergistically with genetic alterations in the PI3K/Akt pathway in both advanced and undifferentiated thyroid carcinoma, implying that aberrant activation of both pathways are critical in thyroid tumorigenesis and tumor progression [25].

2.3 B-Raf^{V600E} and environmental factors

To date there is little published data linking B-Raf^{V600E} with any environmental factor, including radiation exposure. Although multiple population-based cohort studies, including reports and accounts from survivors of the Hiroshima and Nagasake atomic bombs, the Chernobyl nuclear accident, and the Nevada, Novaja Semlja, and Marshall Islands atmospheric nuclear tests have confirmed a striking association between ionizing radiation exposure in childhood/adolescence and the subsequent development of non medullary thyroid cancer [26]. The assumption is that nuclear fallout contains ^{131}I and other isotopes that are associated with an increased risk of thyroid cancer. In addition, early treatment strategies of childhood head and neck cancers allowed several retrospective cohorts to demonstrate a strong relationship between medical thyroid radiation and the subsequent development of adult thyroid cancer [27].

As described above, the incidence of thyroid cancer varies by age, sex, and ethnicity. Though some have proposed links with dietary, hormonal, and/or genetic factors, no studies have truly proven a real association [28, 29]. Co-factors looked at include obesity, tobacco, alcohol, soy products, and reproductive factors [30, 31], to name a few. When looking specifically at B-Raf^{V600E} mutation, Curtin et al. studied melanoma patients, in whom B-Raf^{V600E} is the most frequent point mutation and ultraviolet light the most common risk

factor, but they did not find any correlation between UV exposure and V600E mutation [32]. In another study out of Italy [33], a high incidence of B-Raf^{V600E} mutation was found among patients with PTC in an Italian volcanic region, possibly suggesting a link between B-Raf^{V600E} and some thyroid carcinogen not yet identified in the volcanic soil. Conversely, the authors did not find any link between B-Raf^{V600E} mutation and environmental goitrogens (iodine deficiency and cyanate), suggesting these environmental factors can not trigger B-Raf^{V600E} mutation in PTCs [33].

Since the etiology of thyroid cancer remains uncertain, focus is currently on understanding the disease pathogenesis and developing molecular therapies.

3. PTC and B-Raf^{V600E}

3.1 PTCs larger than one centimeter and B-Raf^{V600E} frequency

As mentioned above, both the incidence of PTC and the detection of B-Raf^{V600E} are increasing. The increasing incidence of newly diagnosed thyroid cancers may reflect not only an improvement in diagnostic ability but also recent changes made to the WHO diagnostic criteria [34]. Most series report a frequency of B-Raf^{V600E} mutation in PTCs between 29% and 83% depending on the prevalence of various PTC subtypes. In one large, multicenter review of classical PTCs (those greater than 1 cm), the frequency of B-Raf^{V600E} ranged from 12% in follicular variants, to 60% in classical PTCs, and as high as 77% in tall-cell variant, which are generally associated with more aggressive clinical behavior and loss of responsiveness to radioiodine [25].

The literature to date describes a controversial relationship between B-Raf^{V600E} and the potential aggressiveness of PTCs. Some authors show a positive correlation between B-Raf^{V600E} and advanced PTCs, characterized by poorer outcomes, stage III-IV disease, extra-thyroidal extension, and lymph nodal metastasis, while others do not [25, 33]. In fact, two recent meta-analyses reported an association between B-Raf^{V600E} and the presence of both extra-thyroidal extension and higher clinical stage, but not with age, sex, or tumor size [25, 35]. Furthermore, the role of B-Raf^{V600E} mutation as a poor prognostic factor has been controversially reported in series with short-term follow-ups. Interestingly, Elisei et al. [36] have recently demonstrated that B-Raf^{V600E} mutation status correlates with the worst outcome (persistent disease and lower survival rate) in a cohort of 102 PTC patients with a long-term follow-up of 15 years. In addition, the B-Raf^{V600E} mutation has been demonstrated to be a poor prognostic factor independent of other negative clinicopathological features.

Importantly, Rodolico et al. compared metastatic PTC lymph nodes bearing the B-Raf^{V600E} mutation with wild-type B-Raf lymph nodes. B-Raf^{V600E} positive nodes were larger in size and exhibited a higher prevalence of extra-capsular invasion [37]. Two additional studies reveal a significant association between B-Raf^{V600E} positive tumors and distant PTC metastases [38, 39]. Conflicting data could stem from a variety of issues including different patient recruitment strategies, epidemiological factors, small numbers of cases, as well as the histological tumor classification performed on B-Raf^{V600E} positive PTCs

Conversely, V600E mutation was not found in medullary thyroid carcinomas, follicular thyroid carcinomas, or benign thyroid neoplasms (adenoma or hyperplasia), suggesting that B-Raf^{V600E} may predict not only a histopathologic diagnosis of PTC, but also serve as a marker for more aggressive phenotypes. Moreover, there is scant data in the literature discussing the presence of B-Raf mutations in follicular thyroid malignancies. Although 12% of follicular variant of PTCs carry B-Raf mutations, none of the few oncocytic follicular thyroid adenomas or carcinomas published to date worldwide have ever tested

positive for a B-Raf mutation. In a recent article, Musholt et al. [40] published his results of a small cohort of 44 consecutive oncocytic thyroid neoplasms that harbored sufficient material for DNA extraction to be subjected to molecular analysis. None of his 44 samples, which consisted of 21 adenomas, 20 follicular carcinomas, and 3 oncocytic PTCs, revealed a sequence alteration in the B-Raf mutation hot-spot (V600E). Specifically, some “true” Hürthle cell carcinomas, i.e., predominantly solid oncocytic tumors composed of large ovoid oxyphilic cells that do not feature PTC-like architecture or nuclear characteristics, carried RET/PTC translocations but again, none carried a V600E mutation.

Finally, B-Raf^{V600E} does not overlap in PTCs with other genetic alterations such as Ras gene family mutations or RET/PTC translocations [7, 41, 42]. And, in contrast to the RET/PTC translocation, B-Raf^{V600E} positive PTCs do not demonstrate a link with radiation exposure [42]. Furthermore, B-Raf^{V600E}, but not a RET/PTC translocation, is found in anaplastic thyroid cancers, suggesting that this mutation may be involved in progression of thyroid cancer from PTCs to poorly differentiated and undifferentiated phenotypes [43–45].

3.2 Micro-PTCs and B-Raf^{V600E} frequency

Micro-PTCs (PTCs less than 1 cm in diameter) are a histological variant of PTC and are generally considered to have a very low risk of progression and/or recurrence. Similar to what is seen in larger tumors, B-Raf^{V600E} is also the most common genetic alteration in micro-PTCs (those less then 10 mm), though with a lower incidence (24%–50%) in most series [33, 46–49]. In addition, an even smaller though not insignificant incidence (17%) of B-Raf^{V600E} was present in micro-PTCs of extremely small size (0.5–4 mm) that were incidentally found in patients who underwent surgery for benign thyroid conditions [50]. Recently, Lupi et al. showed that even in micro-PTCs, B-Raf^{V600E} was significantly associated with extrathyroidal extension, high tumor stage, and lack of tumor capsule [47]. In addition, Frasca and Nucera et al. found that though B-Raf^{V600E} was more frequent in PTCs (45.5%) than micro-PTCs (24.3%), B-Raf^{V600E} was associated with extra-thyroidal extension and advanced stage for both diagnoses [33]. These results suggest that like its larger counterpart, B-Raf^{V600E} may also confer a higher risk of tumor progression and invasiveness versus the wild-type in micro-PTCs, potentially serving as a predictive indicator of aggressive behavior in micro-PTCs. Furthermore, since incidental microcarcinomas can evolve into clinical PTCs, patients with B-Raf^{V600E} positive incidental microcarcinomas may warrant careful clinical follow-up.

3.3 B-Raf^{V600E} in FNA specimens

Since fine needle aspiration (FNA) of thyroid nodule is currently the best diagnostic tool in the clinician’s armamentarium when working up a thyroid nodule, it would be useful if mutational analysis of the FNA could either identify patients with malignancy or select a group of patients with potentially increased risk of poor outcomes. Similar to conventional cytological testing, mutation testing is likely to fail if the tumor cell content of the aspirate is inadequate. Moreover, though each of the above methods has demonstrated decent sensitivity and reliability for detecting the mutation in gross pathology specimens, analyses of cytological specimens and paraffin-embedded tissue samples have yielded discordant results. Traditionally about 10–20% of thyroid nodule FNAs prove to be inadequate for cytological diagnosis with the majority possessing insufficient levels of tumor DNA to undergo nucleic acid preparation, and in another 20% (generally with cytological diagnosis of follicular proliferations), it is not possible to determine with certainty whether the nodule is an adenoma or carcinoma [51]. Ten percent of nodules associated with these nondiagnostic FNAs, and 20% of FNAs with indeterminate findings, are ultimately diagnosed as malignant on histological analysis [51]. Currently, there is a discrepancy among clinicians as to the diagnostic relevance of B-Raf^{V600E} mutation identification in

FNA specimens. There are a number of methods currently available for detecting the B-Raf^{V600E} mutation in FNA specimens from thyroid nodules, including direct sequencing using the colorimetric Mutector™ assay, PCR–restriction fragment length polymorphism analysis, PCR–single strand conformational polymorphism analysis, and real-time, allele-specific LightCycler PCR analysis (which is the most rapid, easiest to perform, and least expensive technique with a specificity of 100% and sensitivity of 53.5%).

The prevalence of B-Raf^{V600E} mutation in thyroid nodules with indeterminate cytological diagnoses is not well defined, with a few small studies publishing prevalence rates ranging from 8% to 16%. In statistical analysis, such small prevalence numbers translate into a low sensitivity of B-Raf^{V600E} mutation analysis for diagnosing malignancy. Tumors typically associated with indeterminate FNA cytology are follicular thyroid cancers, which are not associated with B-Raf^{V600E} oncogene mutations, or follicular variants of PTC, in which the B-Raf mutation is rare. In addition, B-Raf^{V600E} mutation is rarely identified in nondiagnostic FNAs, which can account for up to 20% of all FNA specimens. Most importantly since only half of FNA proven PTCs harbor the B-Raf^{V600E} mutation, negative FNA findings do not exclude the presence of a PTC.

Jin et al. [52] looked at 71 FNA samples grouped according to the preoperative cytologic findings (58 PTCs, 13 non-PTC lesions and 5 indeterminate/suspicious lesions). They used four different methods, including direct sequencing, Colorimetric Mutector Assay, real-time LightCycler polymerase chain reaction (LC PCR) with fluorescence resonance energy transfer probes, and an allele-specific LC PCR with CYBR green 1. B-Raf^{V600E} mutation was detected in the FNA specimens in 31 of 58 (53.4%) PTCs but in none of the 13 cases of non-PTCs lesions. All four detection methods showed similar sensitivity (53.5%) and specificity (100%).

Cohen et al. [53] analyzed 91 FNA samples grouped according to the preoperative cytologic findings (malignant, n = 25; benign, n = 11; and indeterminate, n = 55). In particular, B-Raf^{V600E} mutation was able to be detected in the FNA specimens and confirmed the diagnosis of conventional PTC in 72% of carcinomas within the malignant group, and established the diagnosis of PTC in 16% of carcinomas within the indeterminate group. No B-Raf^{V600E} mutation was detected in the FNA specimens taken from the benign thyroid lesions. These results demonstrate that B-Raf^{V600E} mutation is common in conventional PTCs, and can be reliably detected in cells aspirated from a thyroid nodule suggesting further study of it’s role as marker in the preoperative evaluation of thyroid nodules.

Salvatore et al. [54] looked at 96 cases of PTC for both the B-Raf^{V600E} and RET/PTC mutation in FNA specimens. B-Raf^{V600E} was initially detected in 38% of the samples which on final histopathology were PTC and RET/PTC was found in another 18% of the PTC cases. In total, the identification of either a B-Raf^{V600E} or RET/PTC translocations refined the diagnosis of PTC in five of 15 samples that were considered either indeterminate or insufficient at cytology. No mutation was found in FNA of follicular adenomas or nontoxic nodular goiters.

Given the increasing prevalence of thyroid nodules and the widespread popularity of FNA, even though only a small fraction (7–23%) of patients with ultimately proven PTCs may yield a diagnostic benefit from B-Raf mutational analysis in FNA material modest gains in diagnostic ability could translate into a definite diagnosis for a number of patients. As a separate issue, no studies have yet looked at the potential prognostic value of B-Raf^{V600E} positive FNAs or any potential value in prescribing or altering treatment regimens. Larger prospective studies will be necessary to calculate the diagnostic utility of FNA genome-wide molecular analyses.

4. Current Treatment of DTC

The current standard of care for treatment of differentiated thyroid carcinoma is surgical excision, with either near or total thyroidectomy and central lymph-node dissection, followed by radioiodine treatment in high-risk patients. Patients are prescribed daily suppressive therapy with thyroxine that restores clinical euthyroidism, decreasing the concentration of thyroid stimulating hormone (TSH) to within normal range in moderate-risk patients, or to low levels in high-risk patients (thyroxine suppressive treatment). Follow-up at 9–12 months involves neck ultrasonography and measurement of serum thyroglobulin (Tg) concentration after TSH stimulation to detect persistent or recurrent disease. Subsequent follow-up consists of yearly clinical exams, neck ultrasonography, and serum Tg level [55]. However, at the detection of persistent or recurrent disease, treatment generally entails thyroid hormone suppression, re-operative surgery, and radioiodine treatment if radioiodine uptake is present in neoplastic foci.

4.1 Outcomes of patients with DTC after treatment

The majority of patients with DTC treated with total or near-total thyroidectomy surgery and subsequently with (or without in those patients at low-risk for recurrence) radioactive iodine ablation have an excellent prognosis. With the exception of those individuals harbouring anaplastic carcinoma, 10-year overall survival is 85–90% and most patients are rendered disease-free [55–57]. However, 10–15% of patients with DTC do recur, though in 75% of patients recurrent disease is confined to the neck and the lymph nodes [55]. Only 5% present with distant metastases, either in the lungs (50%), bones (25%), both (20%), or other organs (5%). Ten-year-survival rates after local recurrence range from 49–68%, dropping to 25–42% for those that recur with distant metastases. Neck recurrences alone are responsible for a third of thyroid cancer related deaths. Novel therapeutics are needed, especially for those with recurrent or distant metastatic disease.

5. Compounds inhibiting B-Raf^{V600E}

5.1 Targeted molecular therapies

Recent advances in understanding the molecular changes that take place in tumorigenesis have led to the development of novel therapeutic approaches that are based upon various molecular targets. This strategy builds on the biological principle that tumor growth is dependent upon genetic alterations such as amplification and/or mutation of proto-oncogenes or deletion of tumor-suppressor genes. Therefore, integrative genomic analyses of cancer genomes promise to unveil patterns of genetic alterations linked to the genesis and spread of human cancers [58]. The ultimate goal is to selectively reduce or block tumor growth and metastatic disease. In the treatment of thyroid cancer one current strategy entails interrupting intracellular signal transduction by altering the B-Raf-MAPK or PI3K-AKT pathways, RET, TK, cMet, or EGF receptor. Another popular approach involves interfering with pro-angiogenic factors such as VEGF. Lastly, some researchers are identifying the DNA methylation/deacetylation repression mechanisms implicated in tumorigenesis that induce re-differentiation of thyroid tumors.

By identifying the "driving events" behind individual tumor proliferation, targeted molecular therapies can be developed to halt cancer progression. In the last decade, examples of such therapy include Gleevec (blocks tyrosine kinase domains in specific proteins such as c-kit and bcr-abl), Herceptin (blocks HER2/neu receptor), and Iressa (blocks EGFR tyrosine kinase) [59]. In numerous human cancers, the presence of B-Raf^{V600E} correlates with an increase of cellular proliferation, and decreased response to chemotherapy [60].

Recently, SB-590885, a novel triarylimidazole that selectively inhibits Raf kinases with a greater potency against B-Raf than C-Raf has been tested in vitro [61]. The compound stabilizes B-Raf kinase in an active conformation and has 100 fold greater kinase activity compared to the non-selective Raf and VEGF receptor inhibitor BAY439006, which stabilizes B-Raf kinase in the inactive conformation. King et al. [61] showed that malignant human melanoma tumor cells expressing oncogenic B-Raf when treated with SB-590885, selectively inhibited MAPK activation, proliferation, transformation, and tumorigenicity. In comparison, other similarly treated cancer cell lines, HCT-116 harboring mutant K-Ras alleles and SKMEL2 with mutant N-Ras alleles, displayed variable sensitivities or resistance. Normal human cell lines displayed little to no activity for SB-590885. King's experiments also illustrated a paradoxical increase in phosphorylated ERK, but not cell proliferation, in both normal melanocytes and in melanoma cells expressing wild-type B-Raf after treatment with SB-590885. The mechanism for this event is unknown but may reflect changes in biochemical feedback loops. In contrast, when treated with the MEK inhibitor C1-1040, those same cell lines plus other malignant ones inhibited ERK phosphorylation and cellular proliferation. Collectively, the data intimate the potential usage of direct B-Raf inhibition in treating patients with oncogenic B-Raf driven tumors. Therefore, the experiments from King et al. [61] lead to the conclusion that the presence of B-Raf^{V600E} mutation correlates with sensitivity to the B-Raf inhibitor.

Interestingly, Friday et al. have recently demonstrated that B-Raf^{V600E} disrupts AZD6244-induced abrogation of negative feedback pathways between extracellular signal-regulated kinase and Raf proteins [62]. AZD6244 (ARRY 142886) is a potent and selective MEK inhibitor currently in early clinical trials [62]. In the majority of human cancer cell lines tested by Friday et al., including those with K-ras or non-V600E B-Raf mutations, AZD6244 induced the accumulation of phospho-MEK, an effect not observed in the most sensitive B-Raf^{V600E} containing cells. Accumulation of phospho-MEK in non-V600E-containing cell lines is due to abrogation of negative feedback pathways. The B-Raf^{V600E} mutation disrupts normal feedback inhibition by disrupting interactions between the MAPK inhibitory proteins Spry2 or Spry4 and B-Raf, and results in elevated baseline phospho-MEK expression and interrupts the AZD6244-induced phospho-MEK accumulation [62].

However, the development of highly specific B-Raf^{V600E} kinase inhibitors represents an important step towards treating human cancers that bear this mutation. Recent experiments show a relative therapeutic efficacy of various B-Raf^{V600E} inhibitors both in vitro and in xenograft animal models at low nanomolar concentrations with non-specific action against B-Raf^{V600E}.

Interestingly, recently a novel selective inhibitor of B-Raf^{V600E}, PLX4720, a 7-azaindole derivative has been designed [60]. With an IC_{50} of 13 nM, the compound delineates a new class of kinase inhibitors with marked selectivity in both biochemical and cellular assays. PLX4720 was designed with a unique propyl group that binds to a Raf-selective pocket in the cleft between the N and C lobes of the kinase domain near the hinge region. This overlaps with the ATP-binding site and traps oncogenic B-Raf^{V600E} kinase in a unique conformation. Tsai et al. [60] showed that in vitro, PLX4720 inhibits B-Raf^{V600E} kinase activity at a 10-fold lower concentration than wild type B-Raf in numerous human cancer cell lines (i.e. melanoma, lung and colon cancer). The distinct propyl substitution of the sulfonamide found in PLX4720 is the feature critical for this oncogenic selectivity, which directs an associated alkyl chain into a small RAF-selective pocket.

In addition, although PLX4720 has the ability to bind both the active and inactive B-Raf^{V600E} protein, by decreasing the steric size of the propyl group, the compound has a reduced ability to inhibit the inactive protein. The altered conformation results in an

increased selectivity for the active form of both B-Raf wild-type and B-Raf^{V600E} mutant protein. This supports one of the major differences that distinguish selective inhibitors such as PLX4720 and non-selective compounds such as BAY 43–9006, that have large tail groups, and are only bind to the inactive protein forms. Consistent with the high degree of selectivity, PLX4720 selectively inhibits the B-Raf/MAPK pathway as well as ERK phosphorylation both in vitro and in vivo in melanoma models. In cells lacking oncogenic B-Raf, MAP kinase activity remains unaffected. In melanoma cell lines, Tsai et al. [60] showed that PLX4720 induced cell cycle arrest and apoptosis exclusively in B-Raf^{V600E} positive cells, demonstrating decreased growth rates in comparison to wild-type B-Raf melanoma cells and controls. When tested in B-Raf^{V600E}-dependent tumor xenograft models, orally dosed PLX4720 (at 20 mg/kg and not 5 mg/kg) caused significant tumor growth delays, including tumor regression, with little evidence of toxicity.

Finally, Sherman et al. [63] recently published their results of an open-label, single-group, phase 2 study clinical trial of motesanib diphosphate (AMG 706), a novel oral inhibitor of VEGF receptors, platelet-derived growth-factor receptor, and Kit. In their study of 93 patients with progressive, metastatic and radioiodine-resistant differentiated thyroid cancer, AMG 706 yielded a partial response in 13 patients (14%), stable disease in 67%, and stable disease for 24 weeks or longer in 37% of patients, measured radiographically. The most common side effects were diarrhea, hypertension, and hypothyroidism. Though they tried to correlate the tumor genotype with clinical response, there were too few tumors to reach a conclusion about an association of responsiveness with B-Raf^{V600E} or other genetic alterations that influence the VEGF-signaling pathway.

Here we review both the selective and non-selective anti-B-Raf^{V600E} compounds used in experimental models of human thyroid cancer. One recent paper [64] suggests that many of the initial studies performed looking at the effects of B-Raf^{V600E} inhibitors on thyroid cancer cell lines in vitro and in vivo have used “historical” cell lines many of which have just recently been confirmed to be not only redundant (cross-contaminated), but not of thyroid origin, likely arising from melanoma and primary human colon carcinoma. Drawing conclusions that are specific to thyroid tumors are difficult at this juncture given the need for the primary authors of the previous studies on these xenograft models of thyroid cancer to clarify their data. Nonetheless, it is important to realize that B-Raf^{V600E} is important in thyroid cancer and to look at these previously published studies as proof of principle of the importance of control of this oncogene rather than looking at thyroid specific information. The only studies in the literature that can be found at this time looking at B-Raf^{V600E} inhibitors and thyroid cancer in vivo use what are now likely to be non thyroid cancer cell lines, so it is important to proceed with some caution when looking at these results below. Muddy though these waters are at this point in time, we will try to look at the previous literature best we can in this review.

5.2 Non-selective compounds against B-Raf^{V600E}: with pre-clinical validations in thyroid cancer

The majority of the non selective compounds against B-Raf^{V600E} act through inhibition of various receptors at the stromal and endothelial levels. Most of these effects cause a decrease of pathological angiogenesis, tumor cellular proliferation and metastatic processes (Table 1). Different compounds have been tested using many cross-contaminated and non thyroid origin cancer cell lines (i.e. melanoma and colon cancer cell lines), and rarely some unique thyroid cancer cell lines both in vitro and in vivo models. These compounds include: BAY-43-9006, NVP-AAL881-NX (AAL-881) and NVP-LBT613-AG-8 (LBT-613).

We will concentrate on therapeutics with in vitro or in vivo testing in human thyroid cancer.

BAY-43-9006—BAY-43-9006, or sorafenib [N-(3-trifluoromethyl-4-chlorophenyl)-N'-(4-(2-methylcarbamoyl pyridin-4-yl)oxyphenyl)urea], is a multi-kinase inhibitor that recently proceeded to clinical testing. It is a potent competitive inhibitor of ATP binding in the catalytic domains of C-Raf, wild-type B-Raf, and B-Raf^{V600E} that is not only able to target Raf kinases, but also receptor tyrosine kinases (RTK) including VEGF receptor-2 (KDR) and platelet-derived growth factor receptor B (PDGFR-B) (Table 1). Its anticancer activity is a result of the dual inhibition of Raf signaling plus VEGF receptor-2-mediated and PDGFR-B-mediated tumor angiogenesis. Sorafenib triggers G1-phase arrest that reduces the in vitro proliferation rate of aggressive human thyroid carcinoma cell lines with an IC_{50} of 5 μmol/L. It has proven efficacy in various human tumor xenografts with few side effects that include emesis, diarrhea, and transaminase elevation [65]. Salvatore et al. employed a B-Raf^{V600E} positive xenograft thyroid cancer model (ARO cell line) to show a decrease in proliferative activity and an increase of apoptosis [66]. Kim et al. went one step further by using B-Raf^{V600E} positive anaplastic thyroid cancer xenografts (DRO cell line) [67]. By treating them with high doses (80 mg/kg) of sorafenib, his results demonstrate inhibition of tumor growth and increased animal survival as well as a significant decrease in angiogenesis. This confirms that the drug targets both the Raf kinase pathway as well as VEGF-receptor-mediated signalling [67]. Carlomagno et al. [68] have been demonstrated that sorafenib is also a powerful inhibitor of RET kinase (Table 1). The authors used human papillary (TPC1) and medullary (TT) thyroid carcinoma cell lines, which harbor spontaneous oncogenic RET alleles. Interestingly, BAY 43-9006 inhibited in vitro oncogenic RET kinase activity in both thyroid cancer cell lines, and in vivo after 3 weeks of oral treatment with BAY 43-9006; the volume of tumoral subcutaneous mass of TT cells xenografts mice was significantly reduced versus vehicle-treated mice. Therefore, BAY43-9006 represents a potential therapeutic tool for RET-positive thyroid tumors, in particular for human medullary thyroid carcinomas.

The Food and Drug Administration (FDA) has already approved sorafenib for the treatment of advanced renal cell carcinoma (which is not associated with B-Raf^{V600E}, and the European Commission has granted marketing authorization of sorafenib tablets for the treatment of patients with hepatocellular carcinoma [69]. The drug is currently under evaluation for melanoma and other malignancies including use for patients with iodine non-responsive thyroid cancer [41]. Though Phase I results were encouraging, Phase II studies thus far reveal only minor responses. Based on these preliminary results, sorafenib monotherapy seems to be incapable of inducing the remission of thyroid cancer [41]. Accordingly, although in vitro sorafenib has cytostatic effects and promotes tumor cell death in nude mice xenografts (ARO and DRO cell lines), this is likely due to synergistic anti-angiogenic effects since it inhibits other pro-angiogenic proteins.

AAL-881 and LBT-613—AAL-881 and LBT-613 are isoquinoline compounds that non-selectively inhibit B-Raf^{V600E} along with VEGF receptors. AAL-881 is an inhibitor of both Raf family kinases and KDR, whereas LBT-613 blocks KDR, Raf family kinases, and RET kinase activity (Table 1). It is unclear if the antitumor activity of both LBT613 and ALL881 is caused by their targeting of B-Raf or is caused by their effects through another target. Treatment in vitro with AAL-881 or LBT-613 causes growth inhibition in human thyroid cancer cell lines that harbour RET/PTC-1 (TPC-1) or B-Raf^{V600E} (NPA, FRO and ARO) by impairing cell cycle progression from S-phase to G2-M phase as well as G0–G1 arrest [70]. In addition, Mitsiades et al. [71] demonstrated that AAL-881 markedly decrease MEK and ERK1/2 phosphorylation in vitro studies. In fact, the authors showed that AAL-881 (Table 1) inhibits phosphorylation of MEK and ERK specifically in B-Raf^{V600E} thyroid carcinoma cells (ARO, FRO, NPA, BHP-5, BHP-14, BHP-17, BHP-18 and BHP-19 cell lines) inducing apoptosis by cleavage of caspase-3 in the BHP-14 cell line [71]. This corroborates a similar experiment that used AAL881 to induce apoptosis in human glioma cell lines in

vitro [72]. Collectively, this data implicates the combination of RAF kinase inhibition, together with the decrease of MEK and ERK phosphorylation, as the ultimate cause of a reduction in tumor growth and apoptosis.

Both AAL-881 and LBT-613 show a 10-fold difference in IC_{50} between human thyroid cancer cell lines harbouring wild-type B-Raf (TCP-1) versus B-Raf^{V600E} (NPA, ARO, FRO) [70]. In TPC1 cells, LBT613 shows greater potency, which may reflect its added property of RET kinase inhibition in vitro. However, this cannot be solely due to the combined effect of both kinases since AAL881 has a similar relative potency in TPC1 cells bearing mutant B-Raf lines, even though it has no effect on RET kinase activity in vitro.

Ouyang et al. [70] used human thyroid tumor mouse xenografts models (ARO and NPA cells, that harbour the B-Raf^{V600E} mutation) to demonstrate that tumor growth occurred at a slower rate when treated with AAL-881 and LBT-613 relative to controls. High dose (0.1 mg/g) LBT613 was associated with considerable toxicity while AAL881 was well tolerated. However, at a lower dose (0.025 mg/kg), LBT613 was well tolerated and resulted in stabilization of xenograft tumor size. AAL881 was less effective, though growth of xenografts was significantly diminished. Importantly, these results transpired without inhibition of MEK or ERK phosphorylation, suggesting that the in vivo activity of these two compounds is caused by off-target effects. Furthermore, they compared the activity of both compounds in thyroid cells expressing RET/PTC-1 and in thyroid cells expressing B-Raf^{V600E}. Data showed that both LBT613 and AAL881 were more active against the RET/papillary thyroid carcinoma cell line, implying that the increased potency may be due to the compound's ability to target both RET (especially LBT613) and B-Raf in a double targeting event. Once again, off-target effects seem to play an important role in the compound's non-selective inhibition. It is therefore unclear if the antitumor activity of both LBT613 and ALL881 is caused by their targeting of B-RAF or is caused by their effects through another target.

5.3 Selective compounds against B-Raf^{V600E}: pre-clinical validations

PLX4032—PLX4032 is a structurally distinct analog of PLX4720 that is currently in phase I clinical trials. The compound represents another molecular inhibitor of B-Raf^{V600E}, boasting a similar potency with an IC_{50} of 44 nM [73]. Sala et al. tested PLX4032 in ARO and NPA human thyroid cancer cell lines bearing the B-Raf^{V600E} mutation. He demonstrated that the compound had anti-proliferative but not pro-apoptotic activity [73]. In TPC-1 thyroid cancer cell line harbouring the RET/PTC1 translocation and wild-type B-Raf, PLX4032 showed an approximately 50-fold higher IC_{50} value compared to the cell lines bearing B-Raf^{V600E}, with neither apoptosis nor cell cycle alterations were observed at concentrations up to 10 μmol/L [73]. These results suggest that PLX4032 has specific activity against B-Raf^{V600E} human thyroid cancer cell lines and may well cause regression of tumor growth (Table 1).

PLX4720 and PLX4032 represent the first class of therapeutic molecules that selectively inhibit B-Raf^{V600E}. The aforementioned studies suggest that these two drugs promote selective inhibition of B-Raf^{V600E}, block proliferation, and can cause tumor regression. Importantly, this efficacy is achieved without apparent toxicity to untransformed cells in vitro or in treated mice. With further testing, both PLX4720 and PLX4032 may soon be ready for clinical trial in cancer patients bearing B-Raf^{V600E}-driven tumors. One, if not both compounds may hold promise in treating aggressive or recurrent papillary thyroid carcinomas for those patients that have few other encouraging options.

6. Conclusions

The prognostic and therapeutic implications of basic scientific discovery of the importance of the B-Raf^{V600E}-MAPK pathway in papillary thyroid cancer is another example of the important work of thyroid cancer researchers leading to translation of discoveries made at the bench to the bedside. The B-Raf^{V600E}-MAPK pathway is dysregulated in human thyroid cancer and is crucial for tumor growth, progression, and metastasis. The role of B-Raf in both initiation, maintenance and promotion of aggressive behavior of some thyroid malignancies is becoming clearer. Utility of routine B-Raf^{V600E} analysis in the context of a multigene mutational platform studies on all fine needle aspiration samples of thyroid neoplasm and also on surgically resected thyroid tissue will become clear after systematic and prospective analysis in large multi-institutional studies. Recent commercialization of B-Raf mutational analysis should fairly quickly help clarify the possible role of this mutation on prognostics on a larger scale.

Inhibition of this pathway is a logical target for drug development treatment of thyroid cancer. Though further studies are needed to fully understand the molecular mechanisms of the B-Raf^{V600E}-MAPK pathway involved in the progression and dedifferentiation of thyroid cancer, we have made substantial progress over the past eight to ten years in developing new selective compounds and bringing them to clinical trial. Even though the pharmaceutical compounds tested in today's clinical trials are still showing low efficacy, this is a drastic improvement from ten years ago when no clinical trials existed and little research was being conducted in this area. Most likely, similar to other cancers, combination therapies will ultimately prove most successful in treating aggressive thyroid cancer. Future research will continue to focus on finding selective compounds that have sufficient activity to inhibit B-Raf^{V600E} kinase in patients with advanced thyroid cancers without causing untoward side effects.

Finally, many of human cell lines, harboring B-Raf^{V600E} or other genetic alterations, widely used in the thyroid cancer field for the past twenty years have been recently used to generate xenograft animal models in order to validate pre-clinically different compounds. Importantly, many of them are not only redundant, but likely not of thyroid origin. Therefore, these results emphasize the importance of cell line integrity addressing correctly every investigator to characterize genomic and immunohistochemical profile of every cell line used in own laboratory and then dissect potential molecular mechanisms tissue-specific and B-Raf^{V600E}–dependent, in order to test new and exiting therapeutic compounds useful to carry-out new and effective clinical trials to care patients with refractory or advanced human thyroid cancers.

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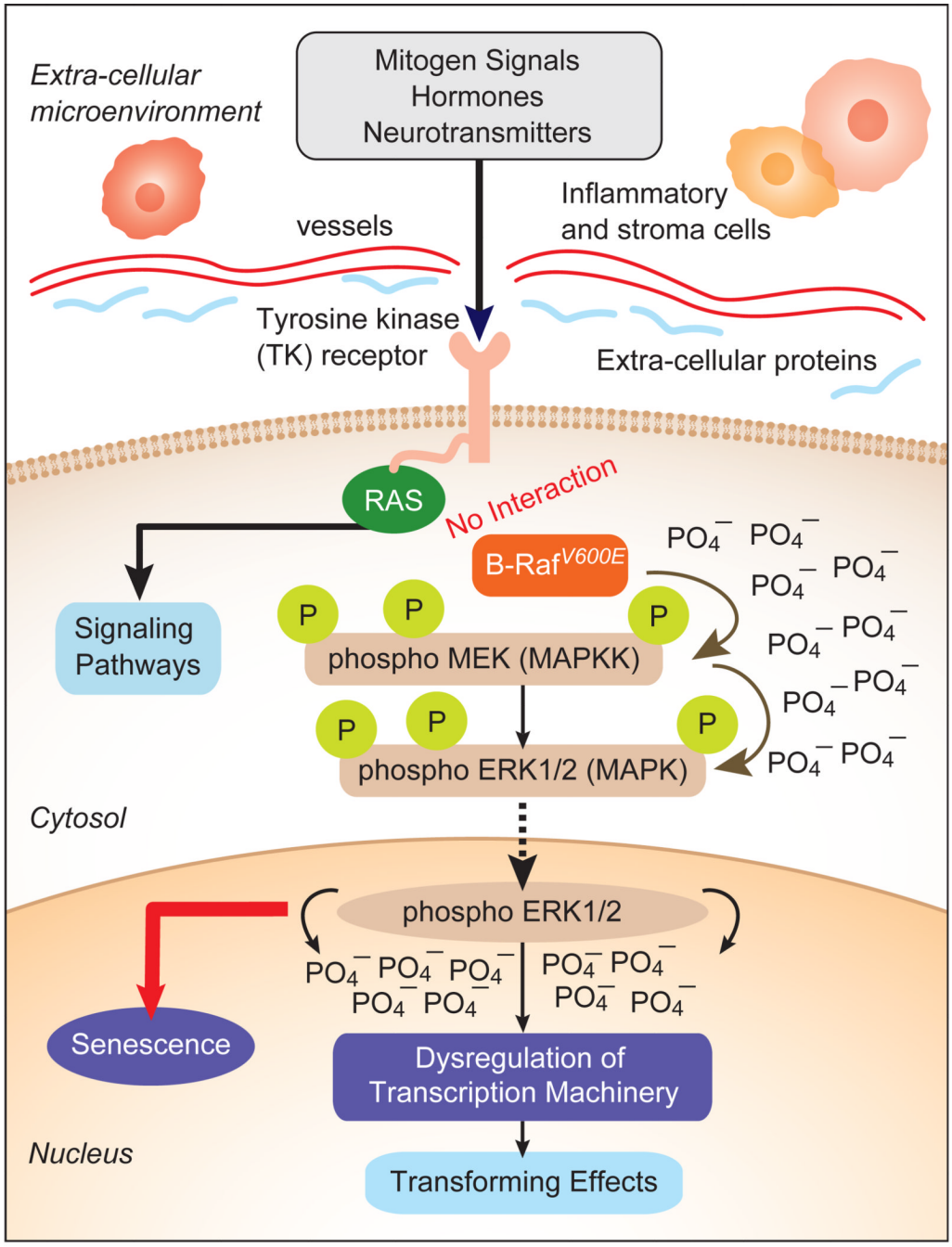

Fig.(1). Physiological mechanism and effects of wild-type B-Raf/MAP kinases signaling pathway

(A) 14-3-3 dimers bind and retain inactive B-Raf protein in the cytosol. **(B)** In response to exogenous stimuli, tyrosine kinase (TK) receptors activate the G protein RAS, which in turn binds the serine-threonine kinase B-Raf after inducing a conformational change by dephosphorylating the N-terminal 14-3-3-binding sites, thus allowing its recruitment to plasma membrane. Activated B-Raf then phosphorylates (PO_4^-) and activates MAPKs/MAPKKs (phospho-ERK1/2), which translocate to the nucleus where they regulate chromatin remodelling and gene expression. Phospho-ERK1/2 then phosphorylates the transactivating domain of various transcription factors within the promoter region of early

and delayed response genes. This process coordinates a highly interconnected and integrated cellular signaling networks that orchestrates the nuclear response to the extra-cellular microenvironment.

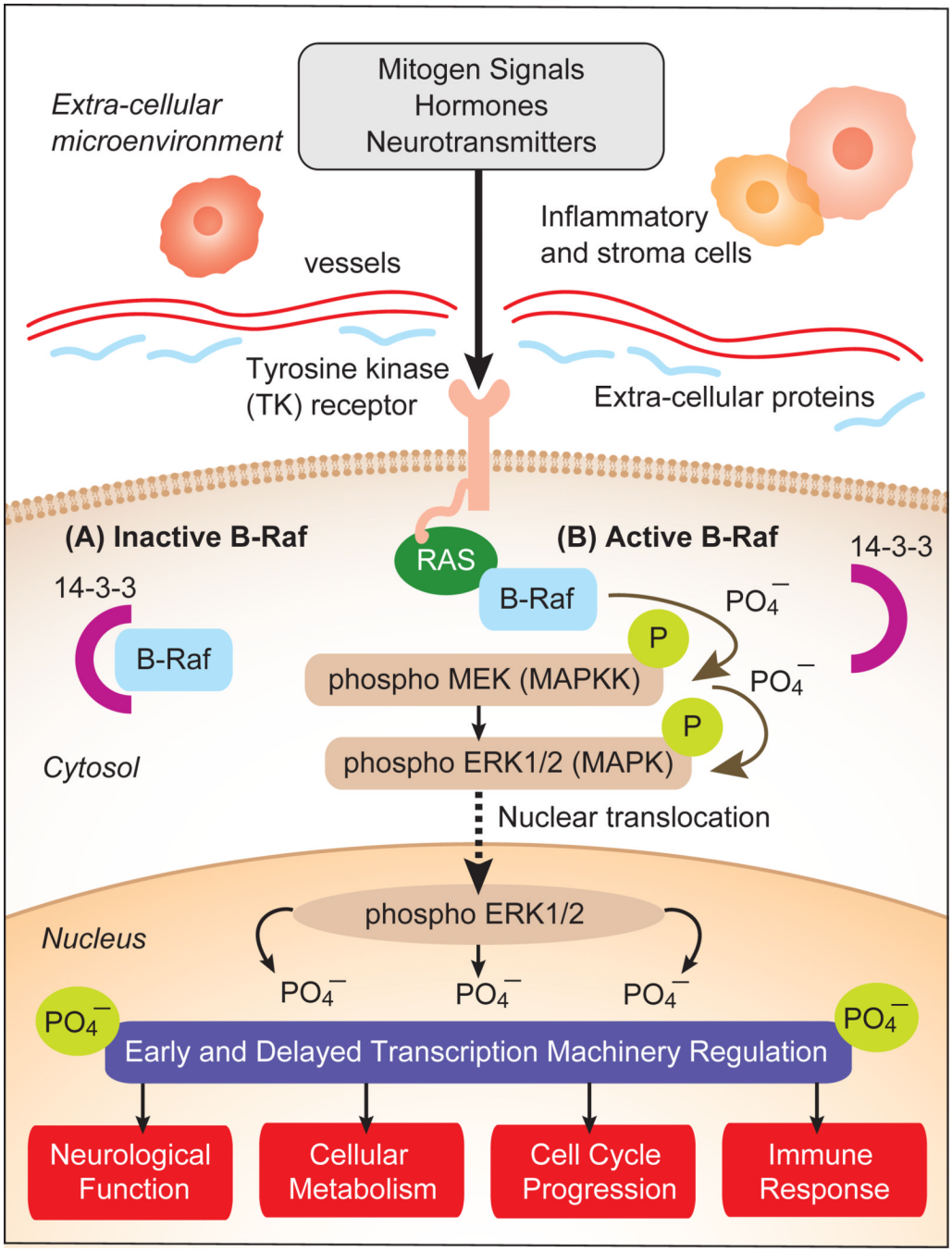

Fig.(2). Mechanism of activation and effects of activated phospho-ERK1/2 signaling pathway by B-Raf^{V600E} oncogene

When the B-Raf^{V600E} oncogene is generated through a point mutation, activation of the MAP kinase pathway becomes constitutive, inducing cells to proliferate indefinitely and initiate tumor formation. B-Raf^{V600E} shows a dramatically increased basal kinase activity (increased phosphorylation, PO_4^-), ~480-folds higher than wild-type B-Raf activity. Response to B-Raf^{V600E} is independent of extra-cellular mitogens and Ras signaling, creating a deregulation of transcription mechanisms. The B-Raf^{V600E}-activated phospho-ERK1/2 pathway transforms human normal cells both *in vitro* and *in vivo*. In addition, B-

Raf^{V600E} induces senescence in normal human foreskin fibroblasts by stimulation of the cell cycle inhibitor p16^{INK4a}.

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Table 1

Biochemical and biological comparisons between selective and non-selective compounds against B-Raf^{V600E} used previously published *in vitro* and/or *in vivo* experimental models of human thyroid cancer.

Compound	Targeted gene	Human thyroid cancer cell line harboring B-Raf^{V600E}	Animal model	B-Raf^{V600E} inhibition (IC_{50})	Biological effects (*in vitro* and in *vivo* studies)	Clinical trial
PLX4032 [a]	B-Raf^{V600E}	NPA*, ARO*	no	126 nmol/L or 205 nmol/L	Cell growth arrest; apoptosis	Phase I
BAY 43-9006 (sorafenib) [b,c,d]	wild-type B-Raf, B-Raf^{V600E}, C-Raf, VEGFR-2 and 3, Flt-3, PDGFR-B, FGFR1, c-KIT, p38α, RET tyrosine kinase	NPA*, ARO*, KAT-4*, 8505c, FRO, DRO*, TPC-1 (harbors RET/PTC-1 and wild type B-Raf), TT (harbors oncogenic RET and wild type B-Raf but not B-Raf^{V600E})	Xenograft by ARO*, DRO* and TT cell lines	5 μmol/L or 1 μmol/L	Cell growth arrest and apoptosis *in vitro* (cytostatic effects); cell growth arrest and apoptosis *in vivo*	Phase II
AAL-881 [e] AAL-881 [f]	wild-type B-Raf, B-Raf^{V600E}, C-Raf, c-ABL, VEGFR2	NPA*, ARO*, FRO BHP-5*, BHP-14*, BHP-17*, BHP-18, BHP-19, NPA*, ARO*, FRO	Xenograft by NPA* and ARO* cell lines no	0.22 μmol/L 5 μmol/L	Cell growth arrest *in vitro* and *in vivo* apoptosis	no
LBT-613 [e]	wild-type B-Raf, B-Raf^{V600E}, C-Raf, RET tyrosine kinase, c-ABL, VEGFR2	NPA*, ARO*, FRO	Xenograft by NPA* and ARO* cell lines	0.21 μmol/L	Cell growth arrest *in vitro* and *in vivo*	no

IC_{50}= concentration of compound that inhibits the kinase activity by 50%.

Abbreviations: FGFR-1, fibroblast growth factor receptor-1; PDGFR, platelet-derived growth factor receptor; VEGFR, vascular endothelial growth factor receptor.

[a]= reference 73;

[b]= reference 66;

[c]= reference 67;

[d]= reference 68;

[e]= reference 70;

[f] = reference 71.

[*] Note that a very recent publication (reference 64) demonstrates that all these cell lines may be in fact melanoma (NPA, DRO, BHP5-16, BHP14-9, BHP-17-10 are genetically identical to each other and the MDA-MB-435S/M14 melanoma cell line) or colon cancer cell lines (ARO and KAT-4 cells are genetically identical to each other and to HT-29 human colon cancer cell line).