In this issue of *Clinical Cancer Research*, Ouyang et al. (1) present evidence suggesting that BRAF is a therapeutic target in thyroid cancer. Previous studies have shown that BRAF is mutated in a high proportion of thyroid cancers, but the therapeutic implications of this have not been investigated. In this article, Ouyang et al. show that small-molecule inhibitors of BRAF can block the growth of thyroid cancer cells expressing mutant BRAF, suggesting that new strategies could be developed to exploit the high rate of mutations in this protein kinase in thyroid cancer.

Thyroid cancer accounts for ~1% of all new cases of cancer each year (~0.5% in men and 1.5% in women). There are ~26,000 new cases of thyroid cancer in America each year with ~2,400 deaths (2) and in Europe there are ~16,000 new cases, with ~3,200 deaths. Thus, thyroid cancer is a relatively rare disease, but it is the most common malignancy of endocrine tissues and, importantly, in the United States its annual incidence has increased by ~50% in the last 25 years (2). Thyroid cancer is divided into four distinct classes: follicular epithelial cell–derived papillary thyroid cancer, which accounts for 80% of cases; follicular thyroid cancer (15% of cases); anaplastic thyroid cancer (2% of cases); and parafollicular C cell–derived medullary thyroid cancer (3% of cases; ref. 3).

Thyroid cancer is not particularly life threatening. Papillary thyroid cancer patients are treated with thyroidectomy and then radioiodine (\(^{131}\)I) is administered to remove residual thyroid tissue and treat metastatic disease. \(^{131}\)I-radioiodine is safe and effective because thyroid tissues preferentially absorb iodine. Finally, the patients receive lifelong thyroxine to suppress production of thyroid-stimulating hormone, which stimulates proliferation of thyroid tissues. Consequently, the majority of papillary thyroid cancer patients can be cured and can expect an average 40-year relative survival rate of 84% (4). Most follicular thyroid cancer patients can also be cured, with an average 40-year survival rate of ~94% (4). Some papillary thyroid cancer patients cannot be cured, however, either because their tumors are inoperable or fail to absorb radioiodine. In addition, in rare cases, papillary thyroid cancer progresses from well differentiated to poorly differentiated or undifferentiated carcinomas and this leads to significantly reduced survival rates. It is also difficult to treat anaplastic thyroid cancer and medullary thyroid cancer, therefore these patients also have significantly reduced survival rates. It is important then to develop novel treatment strategies for patients with incurable thyroid cancer and, to this end, it is important to improve diagnosis, which largely relies on histopathology data and clinicopathologic criteria. These are often incomplete until surgery has been done. To improve both diagnosis and treatment, therefore, it is important to understand the molecular mechanisms that underlie thyroid cancer initiation and progression and to determine how different mutations affect the distinct subtypes of disease. Recent advances have gone some way to achieving this.

The cell signaling pathways that lie downstream of the classic receptor tyrosine kinases have been implicated in human cancer for many years. These pathways normally regulate cellular responses to changing environmental conditions. They are activated when peptide growth factors interact with receptor tyrosine kinases in the cell membrane, bringing about a series of events that lead to activation of several intracellular signaling pathways (5). One such pathway that has been much studied is the RAS-RAF-mitogen-activated kinase/extracellular signal-regulated kinase (ERK) kinase (MEK)-ERK pathway (Fig. 1; ref. 6), which regulates cell proliferation, survival, and differentiation. RAS is a small G protein that is embedded in the inner leaflet of the plasma membrane and, once activated, it recruits the protein kinase RAF to the plasma membrane. RAF is activated at the plasma membrane and it then activates the protein kinase MEK, which, in turn, activates the protein kinase ERK (6). ERK phosphorylates several proteins to regulate gene expression, cytoskeletal rearrangements, and metabolism.

The RAS-RAF-MEK-ERK pathway is hyperactivated in ~30% of human cancers (7), where it provides growth and survival signals. Growth factors can be overexpressed and receptor tyrosine kinases mutated or amplified, thereby stimulating constitutive receptor tyrosine kinase signaling. Similarly, activating mutations in RAS occur in ~15% of human cancers (8). Finally, BRAF, one of the three RAF genes in humans (along with ARAF and CRAF) is mutated in ~7% of human cancers (9, 10). ARAF and CRAF are not commonly mutated in cancer because their regulation is fundamentally different from that of BRAF and therefore they are not predisposed to activating mutations (11).

This signaling pathway also plays an important role in thyroid cancer. The receptor tyrosine kinase RET is not normally expressed in thyroid follicular cells. RET gene

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rarrangements occur in 5% to 30% of papillary thyroid cancers, however, resulting in expression of a fusion protein that contains a constitutively activated RET kinase domain (12). Over 10 different RET rearrangements have been identified and their expression is controlled by the fusion partner promoter. Activating point mutations have also been reported in RAS in up to 80% of thyroid cancers, although the true rate is likely to be considerably lower (13–15). Importantly, recently, it was found that activating point mutations also occur in BRAF in 35% to 70% of papillary thyroid cancers (16–19). Activated versions of BRAF can also be generated by intrachromosomal inversions that fuse the kinase domain of BRAF to the NH_2-terminal portion of AKAP9 (20). These chromosomal aberrations are found in ~11% of patients whose thyroid cancers are thought to be caused by the Chernobyl nuclear power station disaster in 1986, having arisen within 6 years of the accident (20). The BRAF-AKAP fusion proteins are strikingly similar in structure to the RET/papillary thyroid cancer fusion proteins. Thus, activating mutations can occur at several levels in this pathway in thyroid cancer (Fig. 2) and the presence of RAS mutations in up to 85% of microfollicular adenomas (21, 22) suggests that as in melanoma and colorectal cancer, the acquisition of mutations in this pathway in thyroid cancer occurs early and may even be a founder event.

Mutations in RET/papillary thyroid cancer, RAS, and BRAF are mutually exclusive in thyroid cancer (16–19), providing a genetic argument to support the importance of this pathway in this cancer. It also suggests that mutations that activate the pathway at multiple points do not provide a growth advantage or are detrimental to the progression of the cancer, possibly because they lead to excessive signaling. It has also been suggested that the mutations segregate by disease subtype. RET rearrangements and BRAF mutations are thought to occur exclusively in papillary thyroid cancer, whereas RAS mutations do not occur in papillary thyroid cancer and it has been suggested that codons 12 and 61 of HRAS and NRAS are more frequently mutated in follicular tumors and poorly differentiated carcinomas than other types of thyroid cancer (13, 14). Others, however, have not found such a correlation (15). The apparent segregation of particular mutations suggests that individual mutations could be used to distinguish between different forms of thyroid cancer. Large-scale clinical studies are required, however, to confirm these findings.

The most common BRAF mutation in human cancer is a glutamic acid for valine substitution at position 600 (V600E). This mutant is 500-fold activated and is thought to activate BRAF by disrupting an inactive conformation that suppresses its kinase activity in unstimulated cells (23). The cancer most often associated with mutant BRAF is melanoma because up to 70% of patients carry the V600E mutation (9). Consequently, most work has focused on this disease. These studies have established that mutant BRAF stimulates melanoma cell proliferation and survival, showing that BRAF is an oncogene and validating it as a therapeutic target in this skin cancer that is difficult to treat (24, 25). The work of the last 2 years showing that BRAF mutations in thyroid cancer are second only to melanoma in frequency suggests that BRAF may also be an important therapeutic target in this disease. This discovery is one of the most important recent advancements in thyroid cancer research. In particular, the presence of BRAF mutants in anaplastic thyroid cancer of poor prognosis suggests potential for new treatment strategies for incurable thyroid cancer.

To study the clinical potential of targeting BRAF in thyroid cancer, Ouyang et al. (1) have examined how two new RAF kinase inhibitors, ALL881 and LBT613, affect growth and tumorigenicity in conditional rat models of thyroid cancer and also in human thyroid cancer cell lines that express RET/papillary thyroid cancer or mutant BRAF. These investigators previously used RNA interference to show that wild-type BRAF signals downstream of RET/papillary thyroid cancer in thyroid cells (26), suggesting that BRAF inhibition could be effective in tumors that express either oncogene. LBT613 and ALL881 are reasonably toxic, which will hinder their clinical development, but Ouyang et al. use them as molecular tools to study BRAF in thyroid cancer. They show that both compounds inhibit V600EBRAF in vitro and suppress MEK-ERK activity in the rat cell system. However, the ability of these compounds to block MEK-ERK signaling in the human tumor lines is variable. These compounds also suppress expression of ERK-specific phosphatases and, consequently, they can only induce a

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**Fig. 1.** The classic RAS-ERK signaling pathway. Activated receptor tyrosine kinases (RTK) are stimulated by growth factor (GF) binding, leading to RAS activation through a series of adaptor proteins and exchange factors. RAS proteins, which are attached to the inner surface of the plasma membrane, bind to and recruit RAF proteins from the cytosol to the plasma membrane, which is where RAF is activated. RAF phosphorylates and activates MEK, which, in turn, phosphorylates and activates ERK. ERK phosphorylates proteins in the cytosol and also translocates to the nucleus, where it phosphorylates proteins such as transcription factors (TF).
transient suppression of ERK activity in the thyroid cancer cells. Despite these unexpected results, both compounds inhibit thyroid tumor cell proliferation in a dose-dependent manner and, in nude mice, both retard the growth of thyroid tumor xenografts that express mutant BRAF.

Although these data do address the importance of BRAF as a therapeutic target in thyroid cancer, several questions remain. Unfortunately, the structures and full characterization of LBT613 and ALL881 are not presented, making it difficult to compare them with other RAF inhibitors. The most advanced of these is the multitargeting drug, BAY 43-9006 (Sorafenib; Bayer AG, Leverkusen, Germany; ref. 27), which inhibits BRAF activity in vitro, and has some activity in preclinical models of melanoma (24). Clinical trials with Sorafenib monotherapy in melanoma, however, have been disappointing, possibly because the compound does not target BRAF in tumors. Note that its clinical action in renal cell carcinoma seems to be caused by its anti–vascular endothelial growth factor receptor and, therefore, antiangiogenic activity.

It is notable that LBT613 and ALL881 are not particularly potent against V600E BRAF, with IC50 values of 210 and 220 nmol/L, respectively. Thus, they are >5-fold less potent against V600E BRAF than Sorafenib (24) and, although only limited data is presented, it is clear that like Sorafenib, LBT613, and ALL881 are relatively nonselective and inhibit several kinases, including vascular endothelial growth factor receptors. It is unclear, therefore, if the antitumor activity of LBT613 and ALL881 observed by Ouyang et al. (1) is caused by their targeting of BRAF or caused by effects through another target. Importantly, they show that both compounds suppress the growth of tumor xenografts composed of NPA cells, a thyroid cancer line harboring V600E BRAF, but that this occurs without inhibition of MEK or ERK phosphorylation, suggesting that the in vivo activity of these compounds is caused by off-target effects. Furthermore, they show that both compounds are more active against thyroid cells expressing RET/papillary thyroid cancer than cells expressing V600E BRAF and that this may be due to their ability to target both RET (especially LBT613) and BRAF, providing a double-targeting event and once again suggesting that off-target effects are important in the antitumor activity of these compounds.

Despite these uncertainties, this study will provide a framework to encourage further studies into the role played by BRAF in thyroid cancers and the potential for developing new targeted therapies for this disease. Further studies are required, in particular, to use molecular approaches such as RNA interference to assess the role played by oncogenic BRAF in the proliferation and survival of thyroid cancer cells. It will also be interesting to compare LBT613 and ALL881 to agents such as Sorafenib in these thyroid cancer models because Sorafenib does have some antiproliferative and apoptosis-inducing activity in papillary thyroid cancer–derived cells expressing mutant BRAF (28). Finally, it will be interesting to test the activity of MEK inhibitors against thyroid cancer cells (Fig. 2), because as recently shown, these compounds seem to be particularly selective against melanoma cells that harbor BRAF mutations (29).
References


Is BRAF the Achilles' Heel of Thyroid Cancer?

Antonio Chiloeches and Richard Marais


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