Acquired Secondary RAS Mutation in BRAF^{V600E}-Mutated Thyroid Cancer Patients Treated with BRAF Inhibitors

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Background: The BRAF^{V600E} mutation is the most common driver mutation in papillary thyroid cancer (PTC) and anaplastic thyroid cancer (ATC). This mutation is considered actionable and, for BRAF^{V600E}-mutated ATC, a BRAF inhibitor (dabrafenib) in combination with an MEK inhibitor (trametinib) is FDA approved. BRAF inhibitors have also shown efficacy in BRAF^{V600E}-mutated PTC. However, as with all targeted therapies, resistance to these drugs eventually develops. It is essential that we understand the mechanisms of resistance to the BRAF inhibitors in thyroid cancer to develop future strategies to effectively treat these patients and improve survival.
Patients: Herein, we describe four patients with thyroid cancer treated with selective BRAF inhibitors, who developed a RAS mutation in addition to the BRAF^{V600E} mutation at progression.
Results: Patients 1 and 3 acquired a KRAS^{G12V} mutation in the progressive tumor, patient 2 acquired a NRAS^{Q61K} mutation in a progressive lymph node, and patient 4 acquired NRAS^{G13D} mutation on liquid biopsy performed at the time of radiographic disease progression.
Conclusion: Similar to the melanoma experience, the emergence of RAS mutations appears to act as a mechanism of resistance to BRAF inhibitors in thyroid cancers.

Keywords: dabrafenib, anaplastic thyroid cancer, papillary thyroid cancer, differentiated thyroid cancer, clonal divergence, drug resistance, genetic mutations, molecular testing, mutational pathways, kinase inhibitors

Introduction

THE MOST FREQUENT driver mutation detected in thyroid cancer is the BRAF^{V600E} mutation, which occurs in more than 50% of papillary thyroid cancers (PTCs) and in ~45% of anaplastic thyroid cancers (ATCs) (1–6). The BRAF protein is a member of the RAF kinase family. It is usually activated by growth factors via RAS and regulates the mitogen-activated protein kinase factor (MAPK) pathway to induce cell division and/or differentiation (7–9). Activating mutations of the *BRAF* gene occur mostly within the kinase region of the protein and induce its constitutive activity (10).

Therapies specifically targeting BRAF^{V600E} have been explored in BRAF-mutated PTC and ATC (11,12). Dabrafenib is a selective BRAF inhibitor that is FDA approved for BRAF-mutated melanoma, lung cancer, and ATC (13–15), but not for PTC. Vemurafenib is also a selective BRAF inhibitor studied in thyroid cancer but it is only approved for BRAF-mutated melanoma (11). Encorafenib is the most recent FDA-approved selective BRAF inhibitor in combination with binimetinib (MEK inhibitor) for BRAF-mutated melanoma. These drugs have not yet been studied in thyroid cancer.

FDA approval of dabrafenib in BRAF-mutated ATC was based on a phase 2 clinical trial that enrolled 23 patients globally (16). In this trial, patients were treated with a combination of dabrafenib and trametinib, a kinase inhibitor targeting the MEK protein downstream of BRAF. The response rate was 69%. The median progression-free survival (PFS) and overall survival (OS) had not been met at the time of the publication and presentation to the FDA; however, a follow-up abstract presented at ESMO 2018 (17) reported a median PFS and OS of 60 and 86 weeks, respectively.

Although BRAF inhibitors are not approved for PTC, an open-label, nonrandomized phase 2 trial at 10 academic centers worldwide demonstrated antitumor activity in patients with progressive, BRAF^{V600E}-mutated PTC treated with vemurafenib (12). Patients were divided into two groups. Cohort 1 was comprised of patients who had not received vascular endothelial growth factor (VEGFR)-targeted kinase inhibitors (*n* = 26), and cohort 2 included patients who had been previously treated with a VEGFR-targeted kinase inhibitor (*n* = 22). The partial response rate in cohort 1 was 38.5%, median PFS was 18.2 months, and the median OS was not reached. In cohort 2, the response rate was 27.3%, and median PFS and OS were 8.9 and 14.4 months, respectively. The efficacy of dabrafenib

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with or without trametinib was also studied in BRAF-mutated PTC in a large multicenter study. Patients were randomized to receive either single-agent dabrafenib or dual therapy with dabrafenib plus trametinib. The investigators reported similar objective responses in both groups, 42% versus 45%. The PFS was longer in the dual therapy arm (11.4 months vs. 15.1 months) but this was not statistically significant (18). Patients on the single-agent dabrafenib were allowed to cross over to dual therapy but results of the responses in this group of patients were not reported. Finally, a clinical trial using dabrafenib plus lapatinib (HER2/HER3 inhibitor) was recently reported at ASCO 2018 (19). This study enrolled all BRAF-mutated thyroid cancers, some of which had been previously treated with BRAF inhibitors. The authors reported a 58% response rate in differentiated thyroid cancer with a PFS of 18 months. Altogether, these recent data indicate that BRAF inhibitors in combination with other targeted therapies have anti-tumor activity in advanced thyroid cancers, in particular ATC.

However, as with other tumor types, thyroid cancer patients eventually develop resistance to BRAF-directed therapies. Several mechanisms of BRAF/MEK-inhibitor resistance acquired during therapy have been well described in melanoma and colorectal cancer patients. These mechanisms involve genetic and/or epigenetic alterations that reactivate the MAPK and/or the PI3K/AKT pathways. These include alternate *BRAF* gene splicing events, BRAF/BRAF^{V600E} protein amplification, mutations in the PI3K pathway (PIK3CA, PTEN, PIK3R1), mutations in the *MEK1/2* gene, and upregulated expression of the MET, PDGFRβ, or IGF1R receptors (20–27). In addition, acquired mutations in the *RAS* gene family, in particular *NRAS* (17.8%), are often detected (21,26,28).

In thyroid cancers, however, the mechanisms of resistance to kinase inhibitors are less well understood. Acquisition of a *KRAS* mutation has been described in RET/PTC1 cells cultured long term with the anti-VEGFR inhibitor pazopanib (29). Regarding BRAF^{V600E}-mutated cells, resistance to BRAF inhibitors can be induced by epigenetic mechanisms such as increased HER3 receptor expression and activation through an autocrine NRG1 loop (30). Also, mutations altering cell cycle- or DNA damage recognition pathways have been described (31,32). We recently demonstrated that long-term exposure (>5 months) of BRAF^{V600E}-mutated poorly differentiated thyroid cancer (PDTC) cells to 1 μM vemurafenib *in vitro* promoted the emergence of an additional KRAS^{G12D} mutation (33). The presence of both mutations significantly increased the rate of proliferation of the affected cells in comparison with cells harboring the BRAF^{V600E} mutation only.

Confirming these data, a case report in a patient on a BRAF inhibitor for BRAF^{V600E}-mutated PTC who developed a KRAS^{G12V} mutation at progression was recently published (34). Herein, we describe four additional patients with BRAF^{V600E}-mutated thyroid carcinomas who received BRAF-directed therapy for their disease. These four patients developed drug resistance and were found to have acquired secondary RAS mutations in the progressive tumor, some of them different from the recently reported KRAS^{G12D} and KRAS^{G12V}. IRB approval was obtained before data collection.

Case Series

Case 1 is a 60-year-old man diagnosed with PTC who underwent a total thyroidectomy and radioactive iodine

(RAI) therapy 14 years before the initiation of vemurafenib. His primary tumor details are unknown. Ten years after initial diagnosis, he underwent surgical excision of a paratracheal mass with sacrifice of the right recurrent laryngeal nerve. Surgical pathology confirmed PTC in the 4.5-cm mass with extension into surgical margins. A follow-up positron emission tomography-computed tomography (CT) showed no evidence of significant disease. However, six months later, the patient reported a palpable mass in the neck. Fine needle aspirate biopsy (FNAB) revealed bilateral recurrent PTC. The patient came to our institution for a second opinion. Head and neck CT showed extensive disease in the lower neck and paratracheal region with nonspecific bilateral pulmonary nodules. He underwent bilateral neck dissection and esophageal muscularis resection. The surgical pathology confirmed PTC in 7 out of 16 lymph nodes and the mediastinal mass showing columnar features with necrosis indicating an aggressive histopathology. The Ki-67 proliferation index was increased at ~20%. Next-generation sequencing (NGS) analysis detecting mutations in the coding sequence of 134 genes and copy number variations in 47 genes was performed on DNA from the tumor material by the MD Anderson CLIA-certified diagnostics laboratory. The tests revealed a BRAF^{V600E} mutation.

One month later, the patient underwent intensity-modulated radiation therapy with 60 Gy to the postoperative area. Two years later, a chest CT showed a marked increase in the size of the pulmonary nodules. Given the progression, the patient was started on vemurafenib. Four months later, imaging showed significant improvement of the bilateral pulmonary nodules. This response was maintained for more than 2 years, but at 30 months later, a chest CT showed a marked increase in the size of one lung nodule while all other nodules remained stable. The patient continued on vemurafenib. Forty-five months later, progression of the lung nodule from 1.8 to 2.9 cm was evident on a chest CT. FNAB confirmed PTC histology of the progressing lung nodule. NGS was performed on the specimen, identifying the pre-existing BRAF^{V600E} mutation, concomitant with a new KRAS^{G12V} mutation. After 50 months of vemurafenib, the drug was stopped and the patient was started on lenvatinib but he did not respond to this treatment. The patient was subsequently started on dabrafenib plus trametinib and had a partial response, which lasted ~14 months before progression ensued.

Case 2 is a patient who was previously reported elsewhere (35) and therefore will only be described briefly. This man presented with ATC at the age of 60 years. The patient had been treated initially with cytotoxic chemotherapy but had progression of disease and then presented at our institution with a massive tumor arising from the left thyroid lobe, invading the esophagus and encasing the carotid and vertebral arteries. The presence of a BRAF^{V600E} mutation was confirmed on liquid biopsy (VAF 26.4%) by using Guardant360™, as tumor biopsy yielded no viable cells. Other mutations included TP53^{R175H} (VAF 22.3%), EGFR^{G322S} (VAF 1.6%), and BRAF amplification (Table 1). He was started on dabrafenib plus trametinib. He had a rapid clinical response with relief of dyspnea and dysphagia but after four weeks developed a new palpable lymph node in the left neck. The lymph node was biopsied; NGS sequencing was performed as described earlier and was positive for both BRAF^{V600E} and TP53^{R175H} mutations, as well as a new NRAS^{Q61K} mutation (Table 1). Because immunohistochemistry identified a

Table 1. Overview of Missense Mutations in BRAF[V600E] Positive Samples at Baseline and at Progression Under BRAF Inhibitors

	Mutations in baseline sample					Mutations in resistant sample				
Patient	NCBI accession No.	Name	DNA change	Protein change	Variant allele frequency (%)	NCBI accession No.	Name	DNA change	Protein change	Variant allele frequency (%)
1	NM_004333.4	BRAF	c.1799T>A	p.V600E	—	NM_004333.4	BRAF	c.1799T>A	p.V600E	—
						NM_004985.3	KRAS	c.35G>T	p.G12V	
2	NM_004333.4	BRAF[a]	c.1799T>A	p.V600E	26.4	NM_004333.4	BRAF	c.1799T>A	p.V600E	—
	NM_000546.5	TP53[a]	c.524G>A	p.R175H	22.3	NM_000546.5	TP53	c.524G>A	p.R175H	—
	NM_005228.4	EGFR[a]		p.G322S	1.6	—	—	—	—	—
						NM_002524.4	NRAS	c.181C>A	p.Q61K	—
						NM_005157.4	ABL1	c.740A>G	p.K247R	—
3	NM_004333.4	BRAF[a]	c.1799T>A	p.V600E	0.4	NM_004333.4	BRAF	c.1799T>A	p.V600E	—
	NM_000051.3	ATM	c.5956A>G	p.I1986V	—	NM_000051.3	ATM	c.5956A>G	p.I1986V	—
	NM_021960.4	MCL1	c.1051dupT	Nonsense	—	—	—	—	—	—
	NM_000546.5	TP53[a]		p.R273H	0.4	NM_004985.3	KRAS	c.35G>T	p.G12V	—
	NM_005228.4	EGFR[a]		p.R776H	0.2	NM_001626.4	AKT2	c.49G>A	p.E17K	—
	NM_001012331.1	NTRK1[a]		p.H571Y	0.6	NM_198253.2	TERT (promoter)	c.-124C>T	—	—
	NM_004958.3	MTOR[a]		p.V169I	0.3					
4	NM_004333.4	BRAF	c.1799T>A	p.V600E	—	NM_004333.4	BRAF[a]	c.1799T>A	p.V600E	0.3
						NM_002524.4	NRAS[a]	c.38G>A	p.G13D	0.3

[a]Indicates that the mutations were detected on liquid biopsy (cfDNA, Guardant 360).

PD-L1-positive tumor micro-environment, we added pembrolizumab to his treatment regimen. The patient responded to the triple therapy (dabrafenib, trametinib, pembrolizumab, or "DTP") and eventually had a complete resection of his primary tumor followed by adjuvant chemoradiation. The patient remains disease free on DTP and is alive 3 years after his initial diagnosis of ATC.

Case 3 is a 74-year-old woman with reportedly benign thyroid nodules known for 6 years who developed rapid onset hoarseness due to vocal cord paralysis and shortness of breath leading to emergent tracheostomy. Incisional biopsy confirmed an ATC. Staging imaging studies revealed stage IVC disease with tracheal invasion, esophageal invasion, metastases to the lung, liver, bones, and a suboccipital mass. NGS identified the following mutations in her primary untreated tumor: BRAF[V600E], ATM[I1986V], and MCL1 (nonsense). Guardant360 liquid biopsy testing before initiation of therapy showed the following: BRAF[V600E] (VAF 0.4%), TP53[R273H] (VAF 0.4%), EGFR[R776H] (VAF 0.2%), NTRK1[H571Y] (VAF 0.6%), and MTOR[V169I] (VAF 0.3%) (Table 1). She was treated with one dose of bridging chemotherapy (abraxane) while waiting for results of molecular testing. Dabrafenib plus trametinib was initiated after the BRAF[V600E] mutation was identified. She had a dramatic response to therapy within 2 months, with a decrease in size of the primary tumor from 4.3 to 1.1 cm with an almost complete metabolic response and improvement in her distant metastatic disease. This response was maintained for 11 months after initiation of dabrafenib plus trametinib, when she developed recurrence of a suboccipital cutaneous nodule; the remainder of her disease had remained stable. This area was observed but continued to progress (Fig. 1). She then underwent total thyroidectomy, bilateral central node and left lateral neck

dissection, as well as surgical excision of the suboccipital nodule. Final pathology confirmed ATC with squamoid features consistent with ATC in the suboccipital nodule. The NGS of the suboccipital nodule identified the following somatic mutations: BRAF[V600E], KRAS[G12V], TERT 124 C>T, ATM[I1986V], and AKT2[E17K]; no fusions were identified (Table 1). The patient declined chemoradiation, was restarted on dabrafenib and trametinib immediately after surgery, and received one dose of pembrolizumab. She was lost to follow-up but was alive with no evidence of progression 9 months postoperatively and 24 months after her initial diagnosis.

Case 4 is a 50-year-old man who underwent total thyroidectomy for a 4.5-cm PTC, with extrathyroidal extension into skeletal muscle. CLIA-targeted sequencing identified the BRAF[V600E] mutation in his primary untreated tumor. He received postoperative RAI treatment on two occasions 8 months apart with a cumulative activity of 255 mCi [131]I. One year after diagnosis, he was found to have a solitary 2-cm lung metastasis; a biopsy confirmed PTC. He was followed with serial images and 1 year later he developed recurrence in the neck with development of a new 1-cm left tracheoesophageal groove mass. Although it was initially stable for 4 months, it then rapidly progressed to a 4-cm mass with involvement of the trachea and larynx. His lung metastases remained stable. FNAB of the neck mass showed recurrent PTC. He was started on dabrafenib plus trametinib, but one week later developed worsening shortness of breath and stridor requiring tracheostomy. He continued on systemic therapy with several interruptions and dose reductions due to fever, chills, and arthralgia. Restaging exams demonstrated anatomic and metabolic response to therapy. This response was sustained for 20 months, when progression in the neck was noted. Pembrolizumab was added to his regimen, but
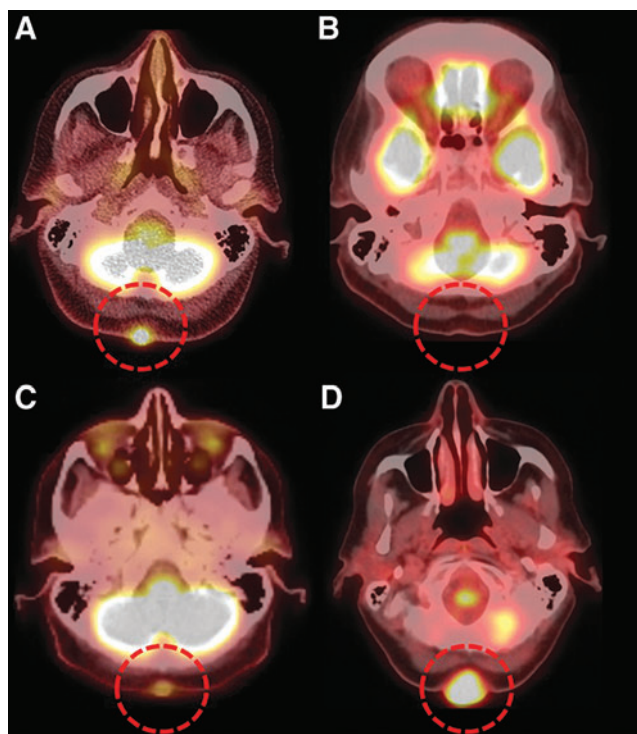
FIG. 1. Patient 3's F18-FDG PET/CT axial image through the neck (**A**) demonstrates the presence of a hypermetabolic midline suboccipital metastatic deposit (red circle). Complete metabolic response with resolution of suboccipital metastasis was seen after 7 months of treatment with dabrafenib and trametinib (red circle, **B**). Tumor recurrence in the suboccipital neck was demonstrated on follow-up F18-FDG PET/CT 3 months later (**C**, red circle) with progression of the metastasis on follow-up imaging (**D**, red circle). FDG, fluorodeoxyglucose; PET-CT, positron emission tomography-computed tomography.

despite three infusions, his neck disease continued to progress (Fig. 2). FNAB of the progressive neck disease showed PTC. A liquid biopsy performed at the time of progression on triple therapy with dabrafenib, trametinib, and pembrolizumab identified BRAF^{V600E} and NRAS^{G13D} mutations (Table 1).

Discussion

BRAF-targeted therapies have been shown to be effective in BRAF-mutated PTC (12) and ATC (16,17), and dabrafenib in combination with trametinib is FDA approved for the latter indication. However, development of resistance to kinase inhibitors presents a significant clinical challenge in the management of solid tumors. In this article, we presented four patients with BRAF^{V600E}-mutated thyroid cancers who progressed while on BRAF inhibitors. Patient 1 and 3 acquired a KRAS^{G12V} mutation in the progressive tumor, while patient 2 acquired an NRAS^{Q61K} mutation in a progressive lymph node, and patient 4 acquired an NRAS^{G13D} mutation as demonstrated by liquid biopsy performed at the time of radiographic disease progression.

Until recently, mutations in the *RAS* and *BRAF* genes were believed to be mutually exclusive, including in thyroid cancers and melanoma (36–38), but other reports found con-

comitant mutations in rare instances, mainly in advanced stages of the disease (39–41). These double events were attributed to heterogeneity of the tumors and emergence of specific tumor cell clones through selective pressure imposed by drug therapy. Data from Raaijmakers *et al.* demonstrated that *NRAS* gene mutations can co-occur with *BRAF* mutations in single melanoma cells during vemurafenib treatment (42). Similarly, a thyroid cancer cell line was shown to harbor both BRAF^{V600E} and KRAS^{G12D} mutations after 5 months of exposure to vemurafenib, and comparison of allelic frequency with that of untreated cells using ddPCR demonstrated that the KRAS^{G12D} mutation was acquired during treatment (33). In addition, a clone of cells harboring both the BRAF^{V600E} and KRAS^{G12D} mutations was isolated from this population, demonstrating that, similar to melanoma cells, mutations in the *BRAF* and *RAS* genes are not mutually exclusive in single PTC cells (43). Therefore, while genetic heterogeneity of the primary tumor and selective pressure provided by the drugs will favor the emergence of a more aggressive tumor cell sub-population, acquisition of additional driver mutations in single tumor cells during therapy will further accelerate clonal evolution.

Expanding our understanding of mechanisms of acquired secondary resistance in BRAF-mutated thyroid cancer cells is vital to improving patient outcomes. Although resistance mechanisms to BRAF inhibitors have been described in other tumors including melanoma, lung adenocarcinoma, and colon cancer patients, events occurring in thyroid cancers might be distinct due to inherent transcriptional and epigenetic differences, and tissue-specific exposure to certain carcinogens.

In melanoma patients who were treated with vemurafenib or dabrafenib, comparison of whole-exome sequencing data from biopsies obtained at baseline and on progression revealed genetic alterations in ~51–58% of the cases (44). These genetic alterations often reactivate the MAPK pathway and are most often detected in the *NRAS* gene (17–20%). These include NRAS$^{Q61K/L}$ (21,42,44–46), NRAS^{T58} (44), and NRAS$^{G12/13}$ (46). Secondary resistance mechanisms through KRAS mutations (KRAS^{G12D} and KRAS^{K117N}) exist in melanoma patients but are rare, and mainly found in xenografts or cell lines (46,47). Other mutations conferring secondary resistance in melanoma are mainly found in the *MEK1/2* (13%) and *PTEN* genes (11%) (44,46).

BRAF-mutated colorectal cancers respond poorly to vemurafenib and dabrafenib, which is possibly due to adaptive feedback reactivation of MAPK signaling mediated by EGFR (48,49). Other acquired resistance to kinase inhibitors are due to wild-type KRAS amplifications (50), RAF dimerization (51), and mutations in the *KRAS* gene (KRAS^{G12R}) (52). Results of a clinical trial combining BRAF and EGFR inhibition with or without MEK inhibition in BRAF^{V600E}-mutated colorectal cancers indicated that 14 out of 29 evaluable patients (48%) developed ≥1 detectable *KRAS* or *NRAS* mutations in cfDNA at the time of disease progression, which were not detectable at baseline. Emerging mutations were KRAS^{G12D}, KRAS^{G12C}, KRAS^{G12V}, KRAS^{G12A}, KRAS^{G13D}, and NRAS^{Q61L} (53).

The BRAF^{V600E} mutation is found in ~2% of lung adenocarcinomas (54), and mechanisms of resistance to BRAF inhibitors in these tumors have only recently been reported. In one case study, Rudin *et al.* described emergence of the
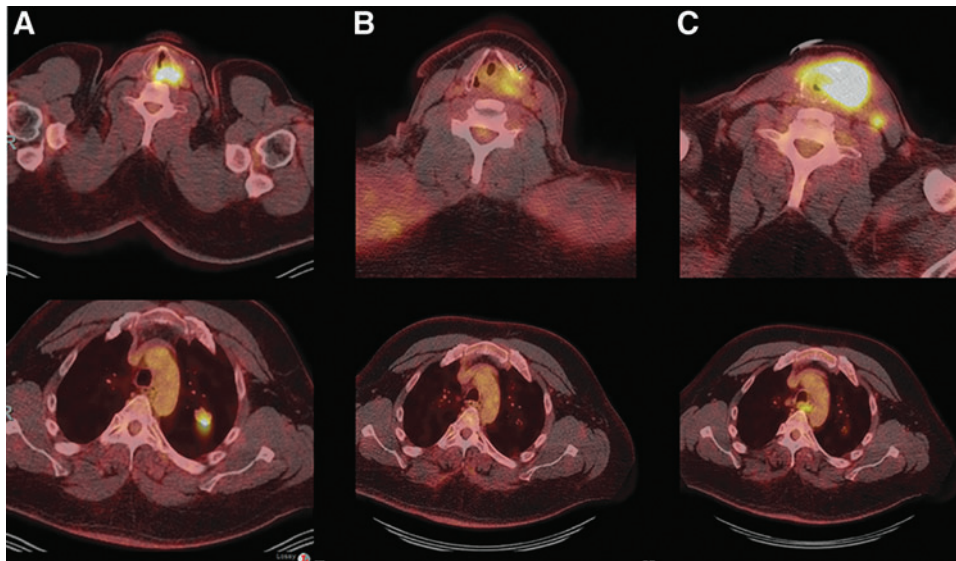
FIG. 2. Patient 4's F18-FDG PET/CT axial image through the neck and chest at baseline (**A**) demonstrated the presence of hypermetabolic tumor with involvement of the trachea and larynx and lung metastases. Restaging exams after 4 months of therapy with dabrafenib and trametinib demonstrated anatomic and metabolic response to therapy with reduction of FDG activity in the recurrence in the left thyroid bed and left central compartment, and pulmonary metastasis has shown resolution of FDG activity and reduction in size (**B**). Pembrolizumab was added to dabrafenib and trametinib at the first sign of disease progression (images not shown). Despite three pembrolizumab infusions, his neck disease continued to progress with an increase in size and FDG uptake of neck tumor and development of new lymph nodes metastases; his lung metastases have remained stable (**C**).

$KRAS^{G12D}$ mutation along with a TP53 mutation on treatment with dabrafenib (55). In another study, resistance to vemurafenib could be overcome with the combination dabrafenib/trametinib, but the acquired mutation(s) were not described (56). A third study identified acquired $NRAS^{Q61K}$ as a possible cause of dabrafenib plus trametinib resistance (57). Altogether, these data combined with ours confirm that $KRAS^{G12}$ is a hotspot mutation emerging in secondary resistance in advanced colorectal, lung, and thyroid carcinomas, but not in melanomas, while $NRAS^{Q61K}$ is found in all four of these tumor types.

In the present article, we describe the emergence of RAS mutations such as $KRAS^{G12V}$, $NRAS^{Q61K}$, and $NRAS^{G13D}$ as mechanisms of resistance in PTC and ATC. Owen *et al.* previously published their case of emergent $KRAS^{G12V}$ mutation at the time of progression on dabrafenib plus trametinib (34). Other proposed mechanisms of acquired resistance in BRAF-mutated thyroid cancers have included epigenetic PI3K/AKT pathway activation through autocrine NRG1-mediated HER3 receptors (30), and autocrine IL-6-mediated JAK/STAT3 pathway activation (58). Duquette *et al.* reported that coexistence of $BRAF^{V600E}$ with either gain of the survival factor MCL1, or loss of the tumor suppressor P16, is associated with primary resistance to vemurafenib in patients with metastatic PTC (31). Further, we recently demonstrated the coexistence of both a $KRAS^{G12D}$ and $BRAF^{V600E}$ mutation in a PDTC cell line, which was associated with cellular dedifferentiation and increase of rate of proliferation (33). The $KRAS^{G12D}$ mutation emerged during long-term treatment with vemurafenib and coincided with resistance to the drug. Interestingly, treating the cells with an MEK inhibitor together with vemurafenib had less of an effect on proliferation than adding a PI3K inhibitor (33).

This is attributed to the fact that these cells rather used the PI3K/AKT pathway, as shown by a significant increase of phosphorylated AKT over phosphorylated extracellular receptor kinase (ERK). Earlier mutagenesis studies have shown that specific RAS mutations may have distinct functional consequences on cell growth (59,60), and evidence now suggests that specific amino acid substitutions at an RAS hotspot have distinct consequences on effector signaling. For example, Ihle *et al.* recently compared downstream signaling in $KRAS^{G12C}$, $KRAS^{G12V}$, and $KRAS^{G12D}$-mutated non-small cell lung cancer (NSCLC) patients and cell lines. They showed increased AKT phosphorylation downstream of $KRAS^{G12D}$ in comparison to $KRAS^{G12C/V}$ that specifically activated RAS-like signaling (61). Thus, similar to the situation in NSCLC, the type of $KRAS^{G12}$ substitution (C vs. D vs. V) occurring in thyroid cancers may drive a specific pathway, which has implications for which drug to introduce at progression, particularly as there is a drug being studied in the clinic that targets $KRAS^{G12C}$ mutations (62).

The MEK inhibitor trametinib is approved in combination therapy with dabrafenib for the treatment of $BRAF^{V600E}$ melanoma. BRAF amplifications, BRAF splice variants, and epigenetic alterations all reactivate the ERK1/2 pathway in presence of BRAF inhibitors alone. However, despite its ability to inhibit proliferation of $BRAF^{V600E}$-mutated melanoma cells or RAS-mutated RET/PTC thyroid cancer cells (29), our study shows that trametinib can be ineffective against emergence of RAS mutations (patients 2, 3 and 4). In melanoma, resistance to BRAF or MEK inhibitors is associated with the induction or persistence of activity of the AKT pathway in the presence of these drugs (63). Mechanisms of resistance to MEK inhibitors in colorectal cancer include alternative MEK1/2 activators such as MAP3K8 (64), high

expression of inflammation-related genes (65), and emerging mutations in MEK1 that either prevent drug binding (24) or activate the gene (25). Mining of TCGA through cBioportal (66) indicated that only 4% of BRAF^{V600E} PTC patient samples had elevated expression of inflammation-related genes such as *IFIT1* and *MX1*, and only a few samples harbored elevated *MEK1/2* or *MAP3K8* expression. No MEK mutations could be detected by NGS in the samples from the patients at progression described in this study. Therefore the mechanisms of resistance to MEK inhibitors in thyroid cancers need to be further investigated.

Summary and Conclusion

We report the emergence of novel KRAS and NRAS mutations in four BRAF^{V600E}-mutated thyroid cancer patients treated with BRAF inhibitors, and suspect that these mutations represent secondary mechanisms of BRAF inhibitor resistance. At this time, the clinical data available are insufficient to make treatment recommendations for these patients, but the type of KRAS amino acid substitution might have an impact on downstream signaling in these tumor cells. Therefore, possible ways to overcome resistance at the present time include the use of selective inhibitors of KRAS^{G12C}, PI3K inhibitors, ERK inhibitors, or immunotherapy combined with BRAF inhibitors. In the future, detailed characterization of the molecular changes in progressive thyroid tumors will point to further therapeutic opportunities, and may identify novel approaches to prevent emergence of drug resistance.

Author Disclosure Statement

Funding Information

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