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Mouse Models as a Tool for Understanding Progression in Braf^{V600E}-Driven Thyroid Cancers

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The development of next generation sequencing (NGS) has led to marked advancement of our understanding of genetic events mediating the initiation and progression of thyroid cancers. The NGS studies have confirmed the previously reported high frequency of mutually-exclusive oncogenic alterations affecting *BRAF* and *RAS* proto-oncogenes in all stages of thyroid cancer. Initially identified by traditional sequencing approaches, the NGS studies also confirmed the acquisition of alterations that inactivate tumor protein p53 (*TP53*) and activate phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (*PIK3CA*) in advanced thyroid cancers. Novel alterations, such as those in telomerase reverse transcriptase (*TERT*) promoter and mating-type switching/sucrose non-fermenting (SWI/SNF) complex, are also likely to promote progression of the BRAF^{V600E}-driven thyroid cancers. A number of genetically engineered mouse models (GEMM) of BRAF^{V600E}-driven thyroid cancer have been developed to investigate thyroid tumorigenesis mediated by oncogenic BRAF and to explore the role of genetic alterations identified in the genomic analyses of advanced thyroid cancer to promote tumor progression. This review will discuss the various GEMMs that have been developed to investigate oncogenic BRAF^{V600E}-driven thyroid cancers.

Keywords: Proto-oncogene proteins B-raf; Thyroid neoplasms; Mice, transgenic

INTRODUCTION

Cancers that originate from thyroid follicular cells, which are the cells in the thyroid responsible for producing thyroid hormones, can be classified into two main groups: differentiated and undifferentiated. The differentiated consist of follicular and papillary, the latter of which account for the vast majority of thyroid cancer cases. In many cases there is a step-wise progression from differentiated thyroid cancers to undifferentiated cancers. The progression is accompanied by a marked difference in prognosis. Most differentiated cancers are well controlled through surgery and radioiodine therapy. By contrast, the undifferentiated cancers do not respond to radioiodine and commonly have metastasis, limiting the effectiveness of surgery. The gain-of-function BRAF^{V600E} is the most common oncogenic driver in thyroid cancers. The canonical signaling pathway activated by BRAF is thought to result in the near exclusive activation of MEK and ERK. Most undifferentiated thyroid cancers are thought to arise from preexisting well-differentiated tumors as a result of acquiring additional alterations. Genomic analysis of advanced thyroid cancers has identified a number of genes that cooperate with oncogenic BRAF to promote thyroid cancer pro-

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gression. Using the genomics of thyroid cancer to guide the development of mouse models of thyroid cancers has led to marked improvement in our understanding of thyroid cancer progression and how the additional alterations that promote thyroid cancer progression might influence their response to therapy. Here we review the genomics of advanced thyroid cancers driven by $BRAF^{V600E}$ and the various genetically engineered mouse models of oncogenic BRAF-driven thyroid cancer.

GENOMICS OF $BRAF^{V600E}$-DRIVEN THYROID CANCERS

In the recent years, several studies in large cohorts of patients, encompassing the full spectrum of human thyroid cancers, from well-differentiated to undifferentiated, have defined the genomic landscape of these tumors [1-5]. These reports provided snapshots of the genetic makeup of papillary thyroid carcinoma (PTC), poorly-differentiated thyroid carcinoma (PDTC), and anaplastic thyroid cancers (ATCs), and gave insights into the key genetic alterations that promote thyroid cancer progression. Mutations in $BRAF^{V600E}$ are tumor-initiating events, occur in roughly half of all thyroid tumors, and are common in both well-differentiated (such as PTC) and advanced cancers (PDTC and ATC). Table 1 and Fig. 1 summarize the crucial genetic events that distinguish PTCs from PDTCs and ATCs. The role of some of these alterations in thyroid cancer progression has been demonstrated *in vitro* and *in vivo* (e.g., tumor protein p53 [*TP53*] loss-of-function, activation of phosphatidylinositol-4,5-bisphosphate 3-kinase [PI3K]/AKT pathway), whereas the precise mechanisms by which others contribute to this process remains to be tested.

Mutations in *TP53* gene, which typically inactivate the p53 protein, are extremely rare events in PTCs, whereas they occur in at least 50% of ATCs. Hotspot mutations in the proximal promoter of the telomerase reverse transcriptase (*TERT*) gene are the second most consistently associated genetic alteration in thyroid cancer progression: they occur in 11% of $BRAF^{V600E}$-mutant PTCs, 44% of PDTCs, and 55% of ATCs. The fact that most of *TERT* mutations in PTCs are subclonal, whereas all of them are clonal events in PDTCs and ATCs suggests that these lesions are selected during thyroid cancer progression. Activating mutations in phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (*PIK3CA*), usually affecting key residues in the protein's helical (E542K, E545K) and kinase (H1047R) domains are strongly associated with $BRAF^{V600E}$-mutant tumors, and track with thyroid cancer progression. The former points to activation of PI3K/AKT pathway as an important process in these tumors, further reinforced by the occasional presence of *AKT1* and *AKT2* oncogenic mutations (e.g., E17K). However, the observation that truncating mutations in phosphatase and tensin homolog (*PTEN*) gene are relatively common in non-$BRAF^{V600E}$ advanced thyroid tumors but mutually-exclusive with their $BRAF^{V600E}$-mutant counterparts suggests that the precise mechanism by which PI3K/AKT pathway is activated

Table 1. The Number of Mutant Samples for Each of the Genes and Pathways for the Indicated Histology Displayed on the Oncoprint (Fig. 1) and Contingency Analysis of These Genetic Events in PTC vs. PDTC vs. ATC

Gene or pathway	No. of $BRAF^{V600E}$ tumors with mutations (%)			Fischer's exacts *P* values		
	PTC	PDTC	ATC	PTC vs. PDTC	PDTC vs. ATC	PTC vs. ATC
$BRAF^{V600E}$	235 (100)	32 (100)	85 (100)			
TP53	1 (<1)	2 (6)	50 (59)	0.0387[a]	<0.0001[a]	<0.0001[a]
TERT promoter	27 (11)	14 (44)	47 (55)	<0.0001[a]	0.3033	<0.0001[a]
PIK3CA	2 (1)	3 (9)	27 (32)	0.0133[a]	0.0165[a]	<0.0001[a]
AKT1/2	5 (<2)	0	5 (6)	>0.9999	0.3209	0.1376
SWI/SNF complex	4 (2)	0	13 (15)	>0.9999	0.0185[a]	<0.0001[a]
NF2	1 (<1)	0	8 (9)	>0.9999	0.1049	0.0001[a]
CDKN2A	0	0	6 (7)	>0.9999	0.1866	0.0003[a]
RBM10	0	0	2 (2)	>0.9999	>0.9999	0.0699
Total	235 (100)	32 (100)	85 (100)			

PTC, papillary thyroid carcinoma; PDTC, poorly-differentiated thyroid carcinoma; ATC, anaplastic thyroid cancer; TP53, tumor protein p53; TERT, telomerase reverse transcriptase; PIK3CA, phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha; SWI/SNF, mating-type switching/sucrose non-fermenting complex; NF2, neurofibromin 2; CDKN2A, cyclin dependent kinase inhibitor 2A; RBM10, RNA binding motif protein 10.
[a]Fisher's exact test significant *P* value.

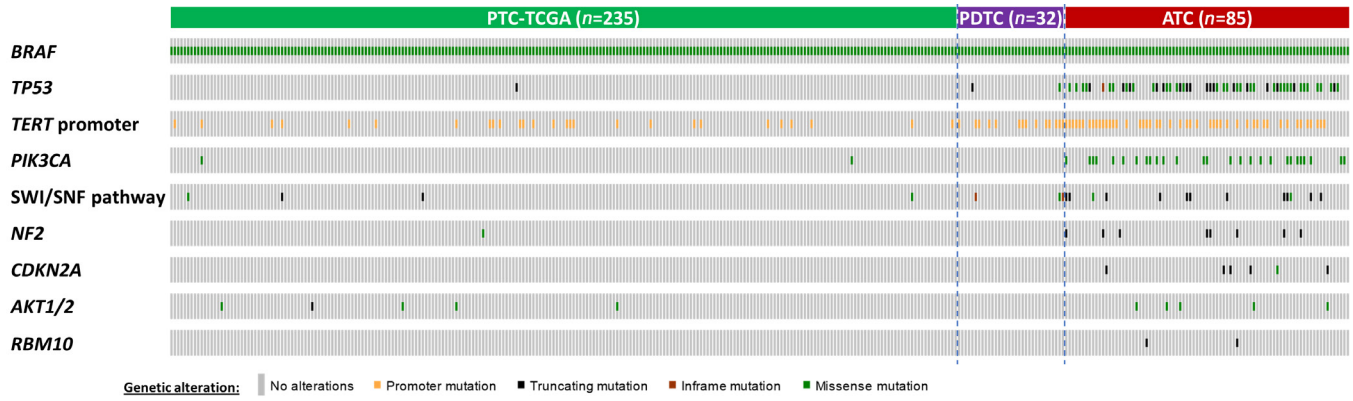

Fig. 1. Genetic alterations in human *BRAF*^{V600E}-driven thyroid cancers. Oncoprint showing the most frequent mutations identified in *BRAF*^{V600E}-mutant of papillary thyroid carcinomas from the The Cancer Genome Atlas study (PTC-TCGA, *n*=235, left), poorly-differentiated thyroid carcinomas (PDTCs, *n*=32, middle), and anaplastic thyroid cancers (ATCs, *n*=85, right). Mutation data compiled from TCGA [1], Kunstman et al. [2], Landa et al. [4], Ibrahimpasic et al. [3], Pozdeyev et al. [5], and Memorial Sloan Kettering Cancer Center Clinical Runs, as of November 1st, 2018. TP53, tumor protein p53; TERT, telomerase reverse transcriptase; PIK3CA, phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha; SWI/SNF, mating-type switching/sucrose non-fermenting complex; NF2, neurofibromin 2; CDKN2A, cyclin dependent kinase inhibitor 2A; RBM10, RNA binding motif protein 10.

matters in tumor biology.

Mutations in members of the SWI/SNF chromatin remodeling complex are significantly enriched in ATCs versus PTCs, both in BRAF^{V600E}-mutant and BRAF wild-type tumors. These are generally mutually-exclusive truncating mutations on genes such as AT-rich interaction domain 1A (*ARID1A*), *ARID1B*, *ARID2*, SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily b, member 1 (*SMARCB1*), and polybromo 1 (*PBRM1*). The exact role of aberrant SWI/SNF function in thyroid cancer biology remains to be tested. Truncating events affecting cell cycle regulator cyclin dependent kinase inhibitor 2A (*CDKN2A*) are also enriched in ATCs, and this is further supported by the frequent copy number losses affecting chr9p21.3 locus, which spans *CDKN2A* gene (not shown). Other less-frequent genetic events, such as truncating mutations targeting neurofibromin 2 (*NF2*) and RNA binding motif protein 10 (*RBM10*) genes, appear to be enriched in BRAF^{V600E}-mutant ATCs as well.

Finally, it is reasonable to think that the generalization of whole-exome and whole-genome sequencing technologies will eventually unmask novel alterations contributing to BRAF^{V600E}-mutant thyroid cancer progression, but these will likely affect a small proportion of cases.

THYROID CANCERS AND THE MAPK PATHWAY

A large number of studies (reviewed in [6]), including the next generation sequencing studies of advanced thyroid cancers described above, have demonstrated that carcinomas arising from thyroid follicular cells have non-overlapping activating mutations of the growth factor receptors RET or neurotrophic receptor tyrosine kinase 1/3 (NTRK1/3), the three isoforms of *RAS* (N, H, and K), or of *BRAF*, which altogether are found in approximately 70% of cases. The fact that all of these alterations activate the mitogen-activated protein kinase (MAPK) pathway highlights its importance in thyroid cancer biology. This has been confirmed through pharmacological and genetic targeting of BRAF and MEK in both preclinical [7-10] and clinical studies [11,12]. As BRAF^{V600E} is the most common alteration that activates the MAPK pathway in thyroid cancers, a number of mouse models of thyroid cancers driven by oncogenic Braf have been developed (Table 2) [8,13-24], providing valuable insight into the biology of thyroid cancer driven by this oncoprotein. In the subsequent sections the various mouse models are discussed.

MOUSE MODELS OF BRAF^{V600E}-INDUCED PTCs

The identification of BRAF^{V600E} in papillary thyroid microcarcinoma (reviewed in [25]) suggests that it is likely an initiating event in thyroid cancer. This hypothesis was first tested by Knauf et al. [13], using the bovine thyroglobulin promoter to drive thyroid-specific expression of a human BRAF^{V600E} cDNA (Tg-Braf). In this study, two mouse lines were created, Tg-

Table 2. Mouse Models of BRAF^{V600E}-Driven Thyroid Cancers

Mouse alleles	Reference	Pros	Cons
PTC			
TG-BRAF	[13,22]	Single transgene	BRAF^{V600E} overexpressed and not under control of endogenous promoter
LSL-Braf^{V600E}/TPO-Cre	[15,19]	Braf^{V600E} under control of endogenous promoter; short latency with near 100% PTC penetrance	Oncoprotein induction at a single time point
TG-CreER^{T2}/Braf^{CA}	[16]	Braf^{V600E} under control of endogenous promoter; flexibility on when oncoprotein is induced	Long latency; leaky Cre activity
TPO-CreER^{T2}/Braf^{CA}	[21]	Braf^{V600E} under control of endogenous promoter; flexibility on when oncoprotein is induced	Long latency
Braf^{CA}/TPO-Cre	[17]	Braf^{V600E} under control of endogenous promoter; short latency with near 100% PTC penetrance	Oncoprotein induction at a single time point
TG-BRAF^{V600E}	[14]	Single transgene	BRAF^{V600E} overexpressed and not under control of endogenous promoter
TG-rtTA/tetO-mycBraf	[18]	Short latency, flexibility on when oncoprotein is induced; can regulate expression of oncoprotein	BRAF^{V600E} overexpressed and not under control of endogenous promoter
TPO-Cre/LNL-BRAF^{V600E}	[23]	Flexibility on when oncoprotein is induced	No tumor formation in absence of elevated TSH; BRAF^{V600E} overexpressed and not under control of endogenous promoter
PDTC/ATC			
TG-CreER^{T2}/Braf^{CA}/Pten$^{f/f}$	[20]	Braf^{V600E} under control of endogenous promoter; flexibility on when oncoprotein is induced	Braf^{V600E} induction and loss of Pten simultaneous which doesn't mimic common human scenario
TG-CreER^{T2}/Braf^{CA}/Pik3ca	[20]	Braf^{V600E} under control of endogenous promoter; flexibility on when oncoprotein is induced	Braf^{V600E} and Pik3ca induction simultaneous which doesn't mimic common human scenario
TPO-CreER^{T2}/Braf^{CA}/Trp53$^{f/f}$	[21]	Braf^{V600E} under control of endogenous promoter; flexibility on when oncoprotein is induced	Braf^{V600E} induction and loss of Trp53 simultaneous which doesn't mimic common human scenario
TPO-Cre/LSL-rtTA$_{ires}$GFP/ tetO-mycBRAF^{V600E}/Trp53$^{f/f}$	[8]	Short latency, flexibility on when oncoprotein is induced; can regulate expression of oncoprotein	BRAF^{V600E} not under control of endogenous promoter
TPO-Cre/LSL-Braf^{V600E}/Trp53$^{-/-}$	[24]	Braf^{V600E} under control of endogenous promoter; short latency	Oncoprotein induction at a single time point; other tumors likely due to germline Trp53 inactivation

PTC, papillary thyroid carcinoma; *TG*, thyroglobulin; *LSL*, lox-stop-lox; *TPO*, thyroid peroxidase; *CreER^{T2}*, Cre/estrogen receptor ligand binding domain fusion; *Braf^{CA}*, Cre-activated *Braf^{V600E}* allele; *rtTA*, reverse tetracycline transcription activator; *tetO-mycBRAF^{V600E}*, tetracycline resistant operator-MYC proto oncogene tagged BRAF^{V600E}; *LNL*, loxP-neoR-loxP; TSH, thyroid-stimulating hormone; PDTC, poorly-differentiated thyroid carcinoma; ATC, anaplastic thyroid cancer; *Pten*, phosphatase and tensin homolog; *Pik3ca*, phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha; *Trp53*, transformation related protein 53; *rtTA$_{ires}$GFP*, reverse tetracycline transcription activator-internal ribosomal entry site–green fluorescent protein.

BRAF2 and Tg-BRAF3. Both lines developed PTCs, albeit with different latency and penetrance, which was attributed to differences in BRAF^{V600E} expression, confirming that oncogenic BRAF alone was able to promote PTC development [13]. Similar results were obtained by Rusinek et al. [14] who generated three different lines with thyroglobulin-driven expression of human influenza hemagglutinin (HA)-tagged human BRAF^{V600E}. In these two models, BRAF^{V600E} was overexpressed and not under control of its endogenous promoter. In addition, expression of the oncoprotein promoted thyroid dedifferentiation resulting in decreased expression of genes involved in thyroid hormone production, which includes Tg. Since the Tg promoter drives expression of the transgenic BRAF in these models, it is likely that expression of the oncoprotein attenuates its own expression, creating a complex negative feedback loop. To overcome this, Franco et al. [15], used lox-stop-lox (*LSL*)-*Braf^{V600E}* mice which contains a Cre-regulated conditional *Braf^{V600E}* knock-in allele. Crossing these mice with thyroid peroxidase (TPO)-Cre mice generated animals with thyroid-specific expression of Braf^{V600E} under control of the endogenous *Braf* promoter, which is expected to begin around embryonic day 14. The resulting *LSL-Braf^{V600E}/TPO-Cre* mice developed PTCs with a near 100%

penetrance at 5 weeks, confirming oncogenic Braf even at endogenous levels is sufficient to promote thyroid cancer. This was confirmed by Charles et al. [16], who crossed mice harboring a Cre-activated *Braf^{V600E}* allele (*Braf^{CA}*) with *Tg-CreER^{T2}* mice to create *Thyro/CreER^{T2}/Braf^{CA}* mice with inducible, thyroid-specific knock-in of Braf^{V600E}. Fourteen days after Braf^{V600E} expression (upon treatment with tamoxifen), thyrocytes displayed a squamous morphology accompanied by a large increase in follicle size, and after 3 months, occasional foci of hyperplastic tall cells were detected, but it was not until 6 months that PTC was detected. The extended tumor latency in the *Thyro/CreER^{T2}/Braf^{CA}* mice compared to *LSL-Braf^{V600E}/TPO-Cre* is likely due to the activation of Braf when animals are adults, whereas it occurs during embryonic development in the *LSL-Braf^{V600E}/TPO-Cre* mice. However, subtle differences between the *Braf^{CA}* and *LSL-Braf^{V600E}* alleles cannot be ruled out.

In all of the models above, thyroid-specific expression of oncogenic BRAF was associated with decreased thyroid function and an increase in serum thyroid-stimulating hormone (TSH) levels, raising the possibility that signaling via the TSH receptor cooperates with oncogenic Braf. Indeed, Franco et al. [15] demonstrated that crossing the *LSL-Braf^{V600E}/TPO-Cre* mice with TSH receptor knockout mice resulted in a marked delay in PTC formation. However, suppression of TSH by high doses of T4 starting shortly after birth, when the thyroid still had a normal histology, was not able to suppress tumorigenesis [15]. The reason for this was not investigated but could possibly be because T4 was administered after the Braf-expressing cells had already become refractory to TSH action. Alternatively, since unliganded TSH receptor retains significant constitutive activity [26], absence of ligand alone may not be sufficient to block the pathway. Consistent with amplified TSH receptor signaling not being required for Braf^{V600E}-induced thyroid tumorigenesis is the observation that focal activation of Braf^{V600E} in the thyroid of *Braf^{CA}* mice by either leaky Cre activity from the Tg-CreER^{T2} allele [16] or thyroid injection of adenovirus expressing Cre recombinase under the control of the thyroglobulin promoter [17] resulted in PTCs despite not elevating TSH.

Expression of BRAF^{V600E} in the immortal differentiated rat thyroid cell line, PCCL3, blocks thyroid differentiation, which can be reversed by inhibition of the MAPK pathway [27,28]. This observation was first confirmed *in vivo* by Chakravarty et al. [18], who demonstrated that thyroid-specific, dox-inducible expression of BRAF^{V600E} blocked thyroid differentiation and generated radioactive iodide (RAI)-refractory PTCs. Consistent with *in vitro* data, this was completely reversible by genetic inhibition of BRAF^{V600E}, and partially by pharmacological inhibition of RAF or MEK [18]. These data ultimately led to a clinical trial testing the MEK inhibitor selumetinib in RAI-refractory thyroid cancers, demonstrating restoration of iodide uptake that was sufficient to justify treating with RAI in eight of 20 patients [29]. Selumetinib restored uptake in all five patients harboring a *NRAS* mutation, but only in one of nine BRAF^{V600E}-positive patients. The difference observed between *RAS*-mutant and *BRAF*-mutant tumors was suggested to be due to incomplete inhibition of the MAPK pathway in the *BRAF*-mutant tumors as a result of higher flux through the pathway. This hypothesis was supported by Nagarajah et al. [19], who demonstrated selumetinib was ineffective at restoring thyroid differentiation in *LSL-Braf^{V600E}/TPO-Cre* mice, but the more potent MEK inhibitor CH5126766 was able to restore differentiation and responsiveness to RAI. CH5126766 is an allosteric inhibitor of MEK that, upon binding, places MEK in an inactive complex with RAF, and thus functions as a RAF/MEK dual inhibitor [30]. In human clinical trials, the RAF inhibitors dabrafenib and vemurafenib, which are more effective than selumetinib in blocking the MAPK pathway, restored iodide uptake sufficiently to justify treating with RAI in six of 10 patients and four of 10 BRAF^{V600E}-mutant patients, respectively [31,32].

THYROID CANCER PROGRESSION AND PI3K PATHWAY

Alterations in effectors of the PI3K pathway are found in approximately 38% of advanced thyroid cancer harboring BRAF^{V600E} (Table 1, Fig. 1). Activating mutations of *PIK3CA* (32%) are the most common, followed by *AKT1* and *AKT2* (Fig. 1) [2,4,5]. While inactivating alterations in *PTEN* are found in advanced thyroid cancer, they are typically associated with activating mutations of *RAS* or inactivation of *NF1*, and are very rarely found in BRAF^{V600E} mutant thyroid cancers [2,4,5]. Charles et al. [20], crossed *Tg-CreER^{T2}/BRAF^{CA}* mice with *Pik3ca^{H1047R}* mice to generate mice with tamoxifen-induced, thyroid-specific knock-in of Braf^{V600E} and Pik3ca^{H1047R}. Tamoxifen-induced expression of Pik3ca^{H1047R} alone produced no thyroid phenotype even 1 year after tamoxifen-induced activation. By contrast, expression of Braf^{V600E} alone produced PTCs 6 to 9 months after BRAF activation. Combining the two oncoproteins hastened Braf^{V600E}-induced PTC formation (PTC detectable 3 to 6 months after tamoxifen), accelerated tumor growth, and after 1 year 70% of the *Tg-CreER^{T2}/Braf^{CA}/Pik3ca^{H1047R}* mice required euthanasia due to labored breathing and thyroid tumors >1 cm^3. Histologi-

cal examination showed 80% of tumors exhibited features consistent with progression to ATCs, which was not seen in tumors expressing $Braf^{V600E}$ alone. Charles et al. [20], further confirmed the accelerated progression of $Braf^{V600E}$-driven thyroid cancers after an alternative intervention for Pi3k pathway activation by crossing *Tg-CreER^{T2}/Braf^{CA}* with $Pten^{flox/flox}$ mice which, like *Pik3ca^{H1047R}*, showed accelerated PTC development and progression to ATC. Shimamura et al. [17] demonstrated that activation of $Braf^{V600E}$ and deleting a single allele of PTEN induced PTCs with undifferentiated foci whereas $Braf^{V600E}$ activation alone produced PTCs without an undifferentiated component. In these experiments, the oncogenic changes were induced by injection of adenovirus expressing Cre under the control of the thyroid globulin promoter into a single thyroid lobe of *Braf$^{CA/+}$/Pten$^{flox/+}$* or *Braf$^{CA/+}$* mice. Very weak Pten staining in the resulting tumors from the *Braf$^{CA/+}$/Pten$^{flox/+}$* mice, which was readily detected in PTCs from *Braf$^{CA/+}$*, suggests that expression from the second Pten allele was suppressed during tumor progression. Consistent with a more aggressive tumor, 67% of the *Braf$^{CA/+}$/Pten$^{flox/+}$* mice were found to have microscopic lung metastasis versus only 22% in the *Braf$^{CA/+}$* mice. The mutually-exclusive occurrence of *BRAF* and *PTEN* alterations in human thyroid cancers limits the usefulness of *Braf*/*Pten* mice to test drug combinations.

In melanomas, activation of the PI3K pathway was shown to promote resistance to RAF inhibitors [33-35]. Currently, it is unknown if this effect exists in $BRAF^{V600E}$-positive thyroid cancer patients. To address this question in an experimental setting, Roelli et al. [10] compared the response of thyroid cancer to the RAF inhibitor PLX4720 in *Tg-CreER^{T2}/Braf^{CA}/Pik3ca^{H1047R}* mice to that of *Tg-CreER^{T2}/Braf^{CA}* mice, demonstrating that activation of the PI3K pathway promoted resistance of the $BRAF^{V600E}$-induced cancers to the RAF inhibitor. In addition, PLX4720 induced paradoxical activation of the MAPK pathway and stimulated tumor growth in *Tg-CreER^{T2}/Braf^{CA}/Pik3ca^{H1047R}* but not *Tg-CreER^{T2}/Braf^{CA}* mice. Combining the PI3K inhibitor GDC-0941 with PLX4720 restored inhibition of the MAPK pathway by the RAF inhibitor, along with suppressing the PI3K pathway, which produced a marked antitumor response [10]. Alternatively, ElMokh et al. [9] demonstrated a potent anti-tumor response in *Tg-CreER^{T2}/Braf^{CA}/Pik3ca^{H1047R}* mice treated with the MEK inhibitor, which was modestly enhanced by the addition of GDC-0941.

Progression of tumors in the *Tg-CreER^{T2}/Braf^{CA}/Pik3ca^{H1047R}* and *Tg-CreER^{T2}/Braf^{CA}*/$Pten^{flox/flox}$ mice to ATCs confirms the conclusion from human genomic data that activation of the PI3K pathway promotes progression of $BRAF^{V600E}$ thyroid cancers. The observation that *Tg-CreER^{T2}/Braf^{CA}/Pik3ca^{H1047R}* thyroid are resistant to RAF inhibitors suggests that $BRAF^{V600E}$-positive human thyroid cancer with activation of the PI3K pathway would also be resistant. Conversely, the marked anti-tumor response to combined RAF and PI3K inhibitors in mice suggests this drug combination maybe effective in the equivalent human thyroid cancers. However, clinical trials in other cancers combining MAPK and PI3K kinase pathway inhibitors found significant toxicity [36] that will likely have to be addressed (i.e., isoform selective inhibitors or schedule optimization) for this to be a viable option for long-term thyroid cancer treatment. PI3K activation was demonstrated to induce resistance to RAF inhibitors. It remains to be determined if resistance induced by activation of the PI3K pathway would extend to the RAF/MEK inhibitor (i.e., dabrafenib/trametinib) combination that recently received U.S. Food and Drug Administration (FDA)-approval for treatment of $BRAF^{V600E}$ ATCs, and whether addition of PI3K inhibitor would improve the efficacy of this combination.

INACTIVATION OF TRP53

As shown in Fig. 1, approximately 59% $BRAF^{V600E}$-driven ATCs harbor alterations in *TP53*, but this combination is very rarely seen in other histotypes of thyroid cancers. Moreover, in the few PTCs identified with alterations in *TP53*, these mutations were subclonal [1]. These results strongly support inactivation of *TP53* as an important step in progression of $BRAF^{V600E}$ PTCs to ATCs. This is supported by three different studies that demonstrated mice with inactivation of transformation related protein 53 (Trp53) and thyroid-specific expression of $Braf^{V600E}$ developed ATCs.

McFadden et al. [21] was the first to report that the combination of $BRAF^{V600E}$ and inactivation of *Trp53* promotes progression to ATCs. To demonstrate this, they crossed thyroid peroxidase promoter driven cre/estrogen receptor ligand binding domain fusion (*TPO-CreER^{T2}*)/*Braf$^{CA/+}$* with *Trp53$^{f/f}$* or *Trp53$^{LSL-R270H/f}$* mice. The resulting *TPO-CreER^{T2}/Braf$^{CA/+}$/Trp53$^{LSL-R270H/F}$* and *TPO-CreER^{T2}/Braf$^{CA/+}$/Trp53$^{f/f}$* mice (referred to as TB270/FL and TBP, respectively) had tamoxifen-inducible, thyroid-specific knock-in of *Braf^{V600E}* and either global expression of mutant Trp53 (*Trp53$^{LSL-R270H}$*) or thyroid-specific deletion of exon 2–10 of *Trp53* (*Trp53$^{f/f}$*). Compared to *TPO-CreER^{T2}/Braf$^{CA/+}$*, the tamoxifen-treated *TPO-CreER^{T2}/Braf$^{CA/+}$/Trp53$^{LSL-R270H/+}$* showed accelerated tumor growth, shorter survival, and histologic features associated with poor prognosis in human PTC

[21]. In addition, 50% of the tumors in older animals showed progression to PDTC or ATC, which was not observed in *TPO-CreER^{T2}/Braf$^{CA/+}$* mice. Homozygous inactivation of Trp53 further accelerated progression to ATC, which was accompanied by a decrease in survival. The authors report a modest but significant decrease in survival of tamoxifen-treated TB270/FL compared to TBP; however, the study was not designed to control for other factors that may contribute to this difference (i.e., tamoxifen dose, animal background).

Zou et al. [24] created mice with thyroid-specific knock-in of *Braf^{V600E}* and global loss of *Trp53* to test the hypothesis that TSH promotes Braf-driven thyroid cancers by suppressing Trp53 and overcoming senescence induced by expression of Braf^{V600E}. To this end, they crossed *TPO-Cre*, *LSL-Braf^{V600E}*, and *Trp53$^{-/-}$* mice to generate the *TPO-Cre*, *LSL-Braf^{V600E}/Trp53$^{-/-}$* (*TPO-Braf^{V600E}-Trp53$^{+/-}$*) and *TPO-Cre/LSL-Braf^{V600E}/Trp53$^{+/-}$* (*TPO-Braf^{V600E}-Trp53$^{-/-}$*) triple transgenic mice. Both mouse lines developed ATCs, with the *TPO-Braf^{V600E}-Trp53$^{+/-}$* having a delay in time to progression of 2 to 3 months.

Knauf et al. [8] created mice with thyroid-specific homozygous loss of Trp53 and dox-inducible expression of BRAF^{V600E} to explore mechanisms of acquired resistance of ATCs to BRAF inhibition. To generate this line, they crossed the following lines *TPO-Cre*, *LSL*-reverse tetracycline transcription activator-internal ribosomal entry site green fluorescent protein (*LSL-rtTA$_{ires}$-GFP*), tetracycline resistant operator-MYC proto oncogene tagged BRAF^{V600E} (*tetO-mycBRAF^{V600E}*), and *Trp53$^{f/f}$* to generate the quadruple transgenic line, *TPO-Cre/LSL-rtTA$_{ires}$GFP/tetO-mycBRAF^{V600E}/Trp53$^{f/f}$* (*BRAF/p53*). Induction of oncogenic BRAF in 4- to 8-week-old *BRAF/p53* mice for 8 weeks produced ATCs in 50% of the mice that rapidly progressed, requiring euthanasia of all ATC bearing mice within 3 weeks of tumor detection.

These three mouse models have differences in ATC latency, which is likely in part due to timing of oncogene activation and Trp53 loss. The ATCs in all three models were very similar to human ATCs, with histological analysis showing pleomorphic giant cells and spindle cells, high mitotic rate, and local invasion. In humans, BRAF^{V600E} ATCs often coexist with a PTC component, which is seen in both *TBP* and *TPO-Braf^{V600E}-Trp53$^{-/-}$* mice. By contrast, a PTC component was not observed in *BRAF/p53* mice. The reason for this difference is unclear, but a possible explanation is the timing of genetic changes. In *TBP* mice, loss of p53 and activation of Braf occur simultaneously in adults, whereas in BRAF/p53 animals, thyroid-specific *Trp53* inactivation occurs late in embryonic development, followed by expression of oncogenic protein when the animals are adults. Similar to human ATCs, in all models, ATC bearing mice had a short survival and lung metastasis were frequently found in both *TBP* (19%) and *BRAF/p53* (50%) mice [8,21]. Frequency of lung metastasis was not investigated in the *TPO-Braf^{V600E}-Trp53$^{-/-}$* mice.

In humans, RAF inhibitors have shown efficacy in BRAF^{V600E} ATC, which is further improved by combining it with MEK inhibitors [11,12]. Indeed, the combination of RAF and MEK inhibitors dabrafenib and trametinib has recently been FDA-approved for the treatment of BRAF^{V600E} positive ATCs [11]. In TBP mice, the RAF inhibitor PLX4720 induced a partial response in the PTC-bearing thyroid gland, but the ATC component continued to grow. There was also a modest extension of survival with the RAF inhibitor with all animals progressing within 35 days after starting treatment. In *BRAF/p53* mice, PLX4720 did not have an effect, and cell lines generated from *TPO-Braf^{V600E}-Trp53$^{-/-}$* mice were resistant to RAF inhibitors. As in humans, treating murine ATCs in both *TBP* and *BRAF/p53* with RAF and MEK inhibitor combination produced a more potent inhibition of the MAPK pathway and marked tumor regression that improved survival. Nevertheless, more than half of the *TBP* animals succumbed to the disease after approximately 70 days of treatment. Time to progression of *BRAF/p53* mice treated with CH5126766 was not determined, but given that after genetic inhibition of BRAF^{V600E},which produced a near complete tumor regression, nearly all animals recurred [8], it is reasonable to think that mice treated with CH5126766 would also progress.

In melanoma patients, multiple mechanisms of acquired resistance to RAF inhibitors as well as RAF/MEK inhibitor have been identified (reviewed in [37-40]). The majority of these reactivated the MAPK pathway. Mechanisms of aquired resistance include overexpression of CRAF or COT1, BRAF^{V600E} amplification, activating mutations in *NRAS*, *KRAS*, *MEK1*, or *AKT1* [34,35,41-43], BRAF^{V600E} alternative splicing, activation of phosphatidylinositol-3-OH kinase or increased expression of receptor tyrosine kinases. Other than a single case report identifying a NRAS^{Q61K} in BRAF^{V600E}-positive PTC with acquired resistance to vemurafenib [44], there is no information on the mechanisms of acquired resistance of *BRAF*-mutant thyroid cancers to RAF kinase inhibition. Knauf et al. [8] investigated mechanisms of acquired resistance to genetic inhibition of Braf^{V600E} in the *BRAF/p53* mouse model. Transcriptome analysis demonstrated that reactivation of the MAPK pathway was common to all recurrences which retained sensitivity to the MEK/RAF inhibitor CH5126766.

Whole exome sequencing identified recurrent focal amplifications of *Met* in 45% of the recurrent tumors as well as an activating mutation of *Hras* [8]. The Met-amplified recurrences overexpressed the receptor as well as its ligand hepatocyte growth factor (Hgf), and were exquisitely sensitive to MET kinase inhibitors, which was required for activation of the MAPK pathway [8]. While it remains to be demonstrated in human thyroid cancers, it seems likely that acquired resistance of $BRAF^{V600E}$-positive thyroid cancers to RAF and RAF/MEK inhibitors will center around the reactivation of the MAPK pathway as seen in melanomas [37-40], which likely explains the improved response of ATCs to RAF/MEK inhibitor combination compared to RAF inhibitors alone [11,12]. It will be important to determine events driving acquired resistance to RAF and RAF/MEK inhibitors in humans, especially for the latter, which is now FDA-approved in $BRAF^{V600E}$-positive ATCs.

The ATCs in the *BRAF/p53* mice are heavily infiltrated with macrophages [8], which are almost universally found in their human counterparts [45]. Macrophage infiltration was not investigated in the ATCs from *TBP* or *TPO-Braf^{V600E}-Trp53$^{-/-}$* mice. However, immune deconvolution of transcriptomic data with CIBERSORT [46] demonstrated a similar increase in macrophages of ATCs from *TBP* to those from BRAF/p53 mice (Fig. 2). Macrophages have been suggested to promote tumor progression by stimulating angiogenesis [47,48] and lymphangiogenesis, enhancing chemoresistance [49-51], promoting distant metastases [52-56], and creating an immune suppressive microenvironment. In human thyroid cancers, macrophage infiltration is associated with decreased survival in both PDTCs and ATCs [45,57]. Depletion of macrophages in mouse models of BRAF-induced PTC attenuated tumor formation and restored a thyroid follicular architecture [58]. The effect of macrophage depletion on ATCs has yet to be investigated in either mouse models or human trials. Results in other cancers suggest that depletion of macrophages alone is unlikely to have profound or sustained anti-tumor effects but is likely to improve the efficacy of chemo-, targeted or immuno-therapies. The recent demonstration that inhibition of PI3Kγ promotes a change in macrophage polarization from M2-like to M1-like, which is associated with a markedly improved response to immune checkpoint blockade [59,60], suggest altering macrophage polarization may be an effective alternative to macrophage depletion. As ATCs are frequently infiltrated with both exhausted T-cells [61-63] and macrophages [45,64], this combination might have efficacy in this aggressive disease.

Human ATCs have been demonstrated to also be infiltrated with programmed cell death 1 (PD1)-positive T-cells, programmed death ligand 1 (PD-L1)-expressing macrophages, and possibly myeloid derived suppressor cells (MDSCs). Expression of PD1 and PD-L1 has been found to be associated with a positive response to anti-PD1/PD-L1 immunotherapies, whereas infiltration of the immune suppressive MDSCs and macrophages would be expected to promote resistance to immune therapies. Currently it has not been reported whether the mouse models of ATC also replicate the T-cell infiltrate, MDSC, PD1, or PD-L1 positivity of human ATCs. In the age of immunotherapy, it will be essential to expand our knowledge of the tumor microenvironment of human and mouse ATCs to guide investigators on how to best use the mouse models to understand the effects of the complex tumor microenvironment in ATCs on the response to immune therapies and improve the efficacy of immune therapies in advanced thyroid cancers.

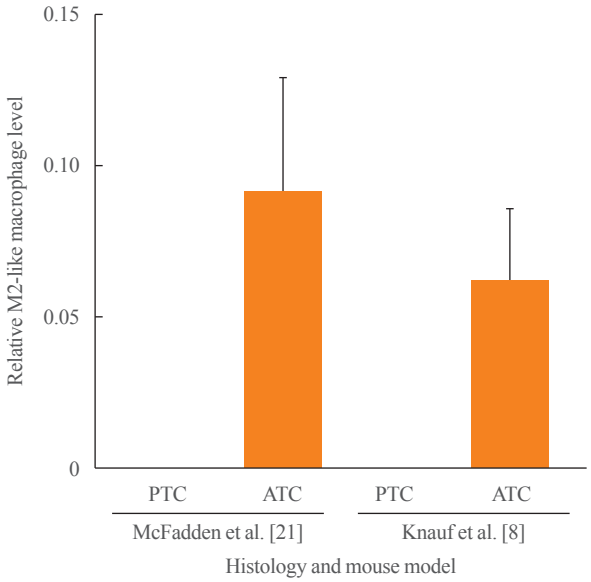

Fig. 2. M2-like macrophage infiltration in oncogenic *BRAF*-driven mouse anaplastic thyroid cancers (ATCs). Immune deconvolution using CIBERSORT [46] was performed on transcriptomic data from the following mouse models: McFadden et al. [21] (GSE55933) *TPO-CreER^{T2}/Braf* (papillary thyroid carcinoma [PTC]) and *TPO-CreER^{T2}/Braf^{CA}/Trp53$^{f/f}$* (ATC). Knauf et al. [8] (GSE118022) PTC, LSL-Braf/TPO-Cre: ATC, TPO-Cre/LSL-rtTA$_{ires}$GFP/tetO-myc-$BRAF^{V600E}$/$Trp53^{f/f}$. Bars show the relative level of M2-like macrophages in the indicated model and cancer histologies. *TPO-CreER^{T2}*, thyroid peroxidase driven cre/estrogen receptor ligand binding domain fusion; *Braf^{CA}*, Cre-activated *Braf^{V600E}* allele; Trp53, transformation related protein 53; LSL, lox-stop-lox; rtTA$_{ires}$GFP, reverse tetracycline transcription activator-internal ribosomal entry site–green fluorescent protein; tetO-myc$BRAF^{V600E}$, tetracycline resistant operator-MYC proto oncogene tagged $BRAF^{V600E}$.

CONCLUSIONS

Genomic analysis of thyroid cancers at different stages of progression has identified several strong candidates for promoting progression of BRAF^{V600E}-driven PTCs to ATCs (Table 1, Fig. 1). Of these, activation of Pik3ca and loss of Trp53 have been demonstrated to be sufficient to drive thyroid cancer progression in mouse models (Table 2). Probably due to its genetic simplicity, there is an excellent concordance between the alterations driving BRAF^{V600E}-mutant progression in thyroid cancer patients and the murine models created to mimic these genetic events. This strongly suggests that results from genomic studies in large cohorts of advanced thyroid cancer patients should inform future approaches involving genetically engineered mouse model research. It also attests to the efficacy of these *in vivo* models to become essential tools to test pharmacological combinations that can be ultimately tested in appropriate clinical trials.

CONFLICTS OF INTEREST

No potential conflict of interest relevant to this article was reported.

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