Activating BRAF and PIK3CA Mutations Cooperate to Promote Anaplastic Thyroid Carcinogenesis

Roch-Philippe Charles1,2, Jillian Silva1,2, Gioia Iezza3, Wayne A. Phillips4, and Martin McMahon1,2

Abstract
Thyroid malignancies are the most common type of endocrine tumors. Of the various histologic subtypes, anaplastic thyroid carcinoma (ATC) represents a subset of all cases but is responsible for a significant proportion of thyroid cancer-related mortality. Indeed, ATC is regarded as one of the more aggressive and hard to treat forms of cancer. To date, there is a paucity of relevant model systems to critically evaluate how the signature genetic abnormalities detected in human ATC contribute to disease pathogenesis. Mutational activation of the BRAF protooncogene is detected in approximately 40% of papillary thyroid carcinoma (PTC) and in 25% of ATC. Moreover, in ATC, mutated BRAF is frequently found in combination with gain-of-function mutations in the p110 catalytic subunit of PI3K (PIK3CA) or loss-of-function alterations in either the p53 (TP53) or PTEN tumor suppressors. Using mice with conditional, thyrocyte-specific expression of BRAFV600E, we previously developed a model of PTC. However, as in humans, BRAFV600E-induced mouse PTC is indolent and does not lead to rapid development of end-stage disease. Here, we use mice carrying a conditional allele of PIK3CA to demonstrate that, although mutationally activated PIK3CAH1047R is unable to drive transformation on its own, when combined with BRAFV600E in thyrocytes, this leads to development of lethal ATC in mice. Combined, these data demonstrate that the BRAFV600E cooperates with either PIK3CAH1047R or with silencing of the tumor-suppressor PTEN, to promote development of anaplastic thyroid carcinoma.

Implications: This genetically relevant mouse model of ATC will be an invaluable platform for preclinical testing of pathway-targeted therapies for the prevention and treatment of thyroid carcinoma. Mol Cancer Res; 12(7); 979–86.

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Introduction
Thyroid cancers derived from follicular epithelial cells are histologically classified into papillary thyroid carcinoma (PTC), follicular or anaplastic thyroid carcinoma (ATC; ref. 1). Although PTC is indolent and readily managed by surgical resection combined with radioiodine therapy, ATC is highly aggressive with more than 50% of ATC patients dying within 1 year after diagnosis (2). Although metastasis is observed in 10% to 20% of patients with ATC, most patients with ATC succumb to locally invasive, inoperable disease that is largely refractory to conventional chemotherapy (3).

Mutationally activated BRAF (commonly T1799→A in exon 15) encoding BRAFV600E is detected in approximately 40% of PTC and 25% of ATC (4). BRAFV600E is a constitutively active protein kinase that activates the ERK1/2 MAPK pathway (5). The importance of mutated BRAF in thyroid cancer maintenance is suggested by responses of patients with thyroid cancer to vemurafenib, a pharmacologic inhibitor of BRAFV600E (6). Moreover, conditional, thyrocyte-specific expression of BRAFV600E in genetically engineered mouse (GEM) models results in PTC (7). However, as in humans, PTC in this model is indolent and does not routinely result in progressively lethal disease. Human ATC displays multiple cooperating mutational events in tumor suppressors and oncopgenes such as TP53 (70%–80%), PTEN (10%–20%), BRAF (25%), H- or KRAS (20%–30%), PIK3CA (15%–25%), and CTNNB1 (60%–65%; ref. 8). Hence, by analogy to other cancer types, it is likely that progression to more aggressive disease is due to cooperative interactions between these various genetic abnormalities. To test this, we generated mice with thyrocyte-specific expression of BRAFV600E in conjunction with expression of mutationally activated PIK3CAH1047R, a constitutively activated form of the p110 catalytic subunit of

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PI3'-kinase α (9). Expression of PIK3CA<sup>H1047R</sup>, which is detected in many cancer types, is predicted to promote elevated PI3'-lipid production leading to activation of AKT protein kinases and other PI3'-lipid effectors in the cell (10). In brief, whereas adult-onset, thyrocyte-specific expression of PIK3CA<sup>H1047R</sup> had no detectable effect on the thyroid, it cooperated dramatically with BRAF<sup>V600E</sup> such that mice developed rapidly lethal ATC. Similar observations were also made with thyrocyte-specific expression of BRAF<sup>V600E</sup> combined with PTEN silencing. Using cultured human thyroid cancer cell lines, we demonstrated that these pathways cooperate to regulate the activity of mTOR and the phosphorylation of 4E-BP1. Hence, we propose that this GEM model of ATC, which recapitulates key features of the human disease, will be useful in understanding thyroid cancer progression and modeling the effects of pathway-targeted therapy in the preclinical setting.

Materials and Methods

Mouse breeding and manipulation

BRAF<sup>CA</sup>, Pik3ca<sup>Lat-1047R</sup>, Pten<sup>lox</sup>, and Thyroglobulin:: CreER<sup>T2</sup> mice were described previously (7, 11; 9, 12). BRAF<sup>CA</sup> mice have been backcrossed in FVB/N in the laboratory for more than 10 generations; all the others have been obtained in C57BL/6 F129–mixed background and crossed in FVB/N since obtained. All the mice considered here are predominantly FVB/N. Thyrocyte-specific activation of CreER<sup>T2</sup> activity was achieved by intraperitoneal injection of 1 mg of tamoxifen dissolved in peanut oil into 4-week-old mice.

Cell lines

The 8505c line was cultured as directed in RPMI complemented with 10% FCS [and validated by short tandem repeat (STR) profiling, performed by Microsynth]. Ocut-2 in DMEM complemented with 10% FCS and nonessential amino acids, STR profiles showed that this cell line was not presenting mouse or human contamination and was of female origin as expected from the literature. The STR profile of Ocut-2 did not present any relevant similarities to any registered cell lines of the American Type Culture Collection.

Histology and immunochemistry of mouse thyroid tissue sections

Animal experiments were carried out in accordance with protocols approved by the University of California, San Francisco (San Francisco, CA) Institutional Animal Care and Use Committee (IACUC). Mice were anesthetized by intraperitoneal injection of 1 mg of tamoxifen dissolved in peanut oil into 4-week-old mice.

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tumors displayed an approximately 60% greater tumor burden compared with BRAF<sup>V600E</sup> alone tumors.

To assess the effects of oncogene expression on tumor histology, thyroid specimens were prepared from normal mice or mice expressing BRAF<sup>V600E</sup>, PIK3CA<sup>H1047R</sup>, or combined BRAF<sup>V600E</sup>/PIK3CA<sup>H1047R</sup> (Fig. 1C and D). Both normal- and PIK3CA<sup>H1047R</sup>-expressing thyroid tissue displayed a characteristic follicular architecture composed of a monolayered cuboidal epithelium of thyrocytes. Indeed, even 12 months after PIK3CA<sup>H1047R</sup> expression, no alteration in thyroid architecture was observed (data not shown).

BRAF<sup>V600E</sup> expression elicited PTC displaying the characteristic papillary thyroid architecture at 6 to 9 months after initiation as described previously (Fig. 1C, middle; ref. 7). In contrast, when BRAF<sup>V600E</sup> and PIK3CA<sup>H1047R</sup> were coexpressed, we observed clear evidence of PTC within 3 to 6 months that extended throughout the entire thyroid (Fig. 1D, left). Moreover, these PTC lesions displayed aggressive characteristics with evidence of invasion into surrounding tissues, most notably the trachea (Fig. 1D, middle). Such invasion resulted in reduced airway diameter that was observed in 70% of the animals represented in the Kaplan–Meier survival curve (Fig. 1A). In addition, the
thyroid tumors in 80% of these animals displayed polygonal giant cells and/or spindle-like cells (Fig. 1D, right), which is a characteristic of human ATC. Such features (local invasion and aberrant tumor cytology) were not observed in PTCs induced by expression of BRAFV600E alone (7).

To further characterize BRAFV600E/PIK3CAH1047R collaboration in this model, we generated Thyro::CreER; BRafCA mice that were either wild-type, heterozygous, or homozygous for the conditional Pik3caLat allele. Of note, 2.5 months after initiation, Thyro::CreER; BRafCA; Pik3caLat/+ mice displayed the expected increase in tumor size compared with mice lacking the conditional Pik3caLat allele. As expected, Thyro::CreER; BRafCA; Pik3caLat/+ mice also displayed an increase in tumor size (≈37%) compared with mice lacking the Pik3caLat allele (Fig. 2A). However, although the difference in tumor size between mice heterozygous or homozygous for the Pik3caLat allele was not statistically significant (Fig. 2B), mice homozygous for Pik3caLat/Lat died more rapidly than their heterozygous counterparts (Fig. 2A) most likely due to an earlier onset of the tracheal invasion.

Human thyroid malignancies are characterized by alterations in marker expression such as galactin-3 (Gal-3) and CK-19 (13). We, therefore, stained BRAFV600E/PIK3CAH1047R–expressing thyroid tumors for expression of these proteins (Fig. 2C). First, we noted that BRAFV600E/PIK3CAH1047R–expressing thyroid tumors displayed areas of PTC and ATC. The regions of PTC were generally positive for Gal-3 and CK-19 as described previously (7). In contrast, regions of ATC retained Gal-3 but lacked CK-19 expression. In addition, ATC-like lesions displayed low/no expression of TTF-1 or E-cadherin, increased Vimentin expression and a higher proliferative index (Ki67).

As an alternative strategy to confirm collaboration of BRAFV600E with alterations in the PI3K pathway, we generated Thyro::CreER; BRafCA; Ptenlox/+ mice to initiate BRAFV600E/PTENnull thyroid tumors. Compared with BRAFV600E alone, these mice displayed increased thyroid tumor burden as soon as 1.5 months after initiation (Fig. 3B). This required complete silencing of PTEN expression because the enhanced tumorigenesis was not noted in Thyro::CreER; BRafCA mice heterozygous for Ptenlox (Fig. 3B). As anticipated, mice with BRAFV600E/PTENnull thyroid tumors developed end-stage disease more rapidly than mice with BRAFV600E expression alone (Fig. 3A, P < 0.01). It was notable that Thyro::CreER; BRafCA+/- mice heterozygous for Ptenlox tended to reach endpoint more rapidly than mice with BRAFV600E expression alone, presumably because of loss-of-heterozygosity of the remaining PtenWT allele (Fig. 3B).

At the histologic level, BRAFV600E/PTENnull thyroid tumors displayed similar characteristics as BRAFV600E/Pik3caLat/+ expressing thyroid tumors displayed areas of PTC and ATC. The regions of PTC were generally positive for Gal-3 and CK-19 as described previously (7). In contrast, regions of ATC retained Gal-3 but lacked CK-19 expression. In addition, ATC-like lesions displayed low/no expression of TTF-1 or E-cadherin, increased Vimentin expression and a higher proliferative index (Ki67).

Figure 2. Thyroid cancer development in mice wild-type, heterozygous or homozygous for Pik3caLat. A, Kaplan-Meier survival curves comparing tamoxifen-treated: 1. Thyro:: CreER; BRafCA; Pik3caLat/+; 2. Thyro::CreER; BRafCA; Pik3caLat/-; or 3. Thyro::CreER; BRafCA+/-; Pik3caLat/Lat mice after tumor induction (+ , P < 0.01; log-rank test). B, tumor burden measured by ultrasonography (+ , P < 0.05; ** , P < 0.01; t test). C, immunofluorescence staining for expression of TTF-1, E-cadherin, Vimentin, Ki67, and Cytokeratin 19 in a histologic section of thyroid from Thyro::CreER; BRafCA+/-; Pik3caLat/- mice focusing on areas of PTC or ATC area as indicated. Nuclear DNA was stained blue with DAPI.
expressing thyroid tumors. Although BRAFV600E/PTENWT thyroids presented expected PTC features after 9.5 months (Fig. 3C), BRAFV600E/PTENnull thyroid tumors displayed a clear PTC phenotype as early as 3.5 months after initiation (Fig. 3D, left). In addition, we also detected evidence of tracheal invasion (Fig. 3D, middle) and progression to ATC (Fig. 3D, right) of BRAFV600E/PTENnull tumors at time points 3.5 to 8 months after tamoxifen induction.

To explore biochemical collaboration between BRAFV600E and PI3'-kinase signaling in more mechanistic detail, we used two human BRAF-mutated ATC cell lines: 8505c and Ocut2 (14). Whereas Ocut2 expresses both BRAFV600E and PIK3CAH1047R, 8505c is BRAF V600E/TP53null. Cells were treated with inhibitors of either MEK1/2 (PD325901; ref. 15) or class I PI3'-kinases (GDC-0941; ref. 16) either alone or in combination with effects on cell proliferation assessed using Crystal Violet staining (Fig. 4A). Although both PD325901 and GDC-0941 displayed antiproliferative effects, in general, MEK1/2 inhibition had a more potent inhibitory effect on cell proliferation than inhibition of class I PI3'-kinases. Moreover, combined blockade of both pathways had a more striking effect on cell proliferation compared with the antiproliferative activity of the single agents.

In parallel with the cell proliferation assays, cells were treated with PD325901 or GDC-0941, either alone or in combination, for 4 or 24 hours at which time cell extracts were analyzed by immunoblotting (Fig. 4B). At both time points, pathway blockade was highly selective in that PD325901-inhibited pERK1/2 with no effect on pAKT- and GDC-0941-inhibited pAKT with no effect on pERK1/
Because these pathways are reported to coordinately regulate the machinery that regulates the initiation of protein synthesis, we assessed the effects of PD325901 and GDC-0941 on the phosphorylation of ribosomal protein S6 (rpS6) and 4E-BP1, that latter of which directly regulates Cap-dependent protein translation (17).

Phosphorylation of rpS6 and 4E-BP1 seemed more sensitive to PI3-kinase inhibition at 4 or 24 hours in Ocut2 cells compared with 8505c cells, which is likely a reflection of the fact that Ocut2 cells express PIK3CAH1047R. Moreover, p-RPS6 and p-4E-BP1 were sensitive to MEK1/2 inhibition in both cell types but this was not observed until 24 hours after inhibitor addition. However, in Ocut2 cells, we detected cooperative effects of combined MEK1/2 plus PI3-kinase inhibition on p-RPS6 at 4 and 24 hours after inhibitor addition. This was also true for p-4E-BP1 24 hours after inhibitor addition but not at 4 hours. We also detected evidence of cooperative inhibitory effects on p-RPS6 and p-4E-BP1 in 8505c cells 24 hours after inhibitor addition, but the effects were not as striking as those observed in Ocut2 cells. Overall, these data suggest that BRAFV600E and PI3-kinase signaling cooperate for sustained thyroid cancer cell proliferation and that mutational activation of PIK3CA may predict for more potent antiproliferative and signaling effects following inhibition of class I PI3-kinases.

Discussion

Anaplastic thyroid carcinoma is a difficult disease to treat because it is generally inoperable and resistant to current regimens of chemotherapy. Although there are provocative hints from small numbers of patients from the phase I vemurafenib clinical trial, it remains to be unequivocally demonstrated that BRAF mutational status is prognostic for therapeutic benefit from BRAFV600E inhibitors. However, the potential clinical benefit of vemurafenib might be influenced by altered signaling through ERBB receptors for EGF ligands as demonstrated by others (18). Furthermore, it was recently demonstrated that blockade of BRAFV600E signaling with a MEK1/2 inhibitor (AZD6244) can restore the expression of the sodium-iodide symporter (NIS), and thereby enhance the efficacy of radiiodine therapy in both GEM models and in patients (19, 20). These data, therefore, suggest that combined pathway-targeted and conventional radiotherapy may be a successful strategy for treating late-stage thyroid cancer.
There are a large number of inhibitors of BRAF<sup>V600E</sup> and PI3<sup>3-K</sup>-kinase signaling being developed for the treatment of a wide range of human cancers. However, it remains unclear whether mutational activation of BRAF, PIK3CA, or silencing of PTEN will serve as strong prognostic biomarkers for thyroid cancer patient responses to pathway-targeted therapy, in part because of effects of these agents on intracellular feedback signaling (18). Indeed, although BRAF mutation is prognostic of response in melanoma, that is not true for patients with colorectal cancer (21, 22). The availability of GEM models of a wide range of thyroid cancer types, including the new model of ATC based on a foundation of mutational activation of BRAF plus either mutation of PIK3CA or silencing of PTEN described here, will allow preclinical modeling of combination pathway-targeted, conventional or immunomodulatory cancer therapy. It was notable that expression of mutationally activated PIK3CA<sup>H1047R</sup> in thyrocytes was largely without effect in the mouse but that it cooperated strongly with mutationally activated BRAF<sup>V600E</sup>. This is not without precedent because PIK3CA<sup>H1047R</sup> expression fails to initiate tumorigenesis in melanocytes and in the lung and pancreatic epithelium (23–25). In contrast, mutationally activated BRAF<sup>V600E</sup> is sufficient to initiate tumorigenesis in all of the above target tissues. Hence, we propose that mutational activation of BRAF<sup>V600E</sup> is a potent initiator of tumorigenesis that is also frequently required for tumor maintenance. In contrast, mutationally activated PIK3CA<sup>H1047R</sup> is a weak initiator of tumorigenesis but greatly promotes tumorigenesis initiated by other oncogenic events (25). This may explain why, to date, pathway-targeted inhibition of PI3<sup>3-K</sup>-kinase signaling has not proven successful in patients, even those whose tumors are silenced for PTEN or mutated for PIK3CA.

TRP53 is also frequently mutated in anaplastic thyroid carcinoma (50% of the cases). Indeed, separately, McFadden and colleagues have demonstrated that combined expression of BRAF<sup>V600E</sup> with silencing of TP53 also elicits anaplastic thyroid carcinoma (26). It has also been shown that the combination of TRP53 and PTEN knockouts could lead to ATC in another mouse model (27). This model has a similar latency to the model described here even if it used an embryonic onset of the cre-recombinase (TPO-Cre). This shows that even if cannot exclude p53 alteration in the BRAF<sup>V600E</sup> PIK3CA<sup>H1047R</sup> model, subsequent p53 alteration resulting in ATC progression is very unlikely.

The cooperation observed between BRAF<sup>V600E</sup> and PI3<sup>3-K</sup>-kinase signaling in GEM models was recapitulated, at least in part, by analysis of <i>bona fide</i> human ATC-derived cell lines. It was clear that combined inhibition of both BRAF<sup>V600E</sup> and PI3<sup>3-K</sup>-kinase signaling had more potent antiproliferative effects than that of single-agent inhibition. Moreover, regulation of key nodes in protein translation, most notably the phosphorylation of 4E-BP1 was clearly under the dual control of both pathways, suggesting that efficient Cap-dependent protein synthesis requires inputs from at least two pathways in ATC-derived cell lines. Although there was a suggestion that the PIK3CA<sup>H1047R</sup>-expressing Ocut2 cells were more sensitive to PI3<sup>3-K</sup>-kinase, an extended analysis of a larger panel of thyroid cancer cell lines will be required to confirm if there is a genotype–drug response phenotype in this disease.

In conclusion, we describe a new mouse model of anaplastic thyroid carcinoma that recapitulates key features of the genetics and pathobiology of the cognate human disease. We propose that this GEM model will be useful for future preclinical studies and to understand the mechanisms by which these pathways cooperate to promote progression of thyroid cancer from an indolent to a lethal disease.

Disclosure of Potential Conflicts of Interest

M. McMahon has received commercial research grant from Novartis, GlaxoSmithKline, and Plexiscon, and is a consultant/advisory board member of Abbvie, Sutro Biopharma, and Igenica Inc. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions

Conception and design: R.-P. Charles, M. McMahon Development of methodology: R.-P. Charles Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): R.-P. Charles, J. Silva, W.A. Phillips Analysis and interpretation of data (e.g., statistical analysis, bioinformatics, computational analysis): R.-P. Charles, J. Silva, G. Iezza, M. McMahon Writing, review, and/or revision of the manuscript: R.-P. Charles, M. McMahon Study supervision: M. McMahon

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