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Modeling anaplastic thyroid carcinoma in the mouse

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Abstract

Anaplastic thyroid carcinoma is the least common form of thyroid cancer; however, it accounts for the majority of deaths associated with this family of malignancies. A number of genetically engineered immunocompetent mouse models recapitulating the genetic and histological features of anaplastic thyroid cancer have been very recently generated and represent an invaluable tool to dissect the mechanisms involved in the progression from indolent, well differentiated tumors to aggressive, undifferentiated carcinomas, and to identify novel therapeutic targets. In this review, we focus on the relevant characteristics associated with these models and on what we have learned in terms of anaplastic thyroid cancer biology, genetics, and response to targeted therapy.

Introduction

Anaplastic thyroid cancer (ATC) is one of the most aggressive human neoplasms, with a median survival of less than 6 months after diagnosis [39]. While ATC might constitute only 5% of all thyroid malignancies, it accounts for the majority of thyroid cancer deaths [10, 41]. As this cancer presents as a rapidly growing tumor mass in the neck area, eradication surgery is one approach to control its progression. However, this approach commonly fails due to invasion into the surrounding tissues [10]. In fact, metastasis is not the main cause of death in ATC, and most patients die from the locally invasive tumor, which compromises airway passages [36]. Although there have been reports of cases successfully treated with multimodal therapeutic approaches, much more research is needed in this area, as ATC is almost invariably refractory to treatment [59, 49].

It is now accepted that ATC often arises from pre-existing, less aggressive, well-differentiated thyroid carcinomas (DTC), namely papillary thyroid cancer (PTC) and follicular thyroid cancer (FTC), through a process of de-differentiation, where epithelial and thyroid differentiation markers are lost [27, 39]. Histologically, ATC presents as a solid mass composed of large pleomorphic and bizarre giant cells, spindle cells, or squamoid cells, accompanied by areas of necrosis and hemorrhage [39]. The presence of many mitotic figures and atypical mitoses is also observed, which points to the high rate of cell proliferation in these tumors [1, 39].

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The rarity of ATC has been for a long time a major obstacle in the study of the mechanistic features responsible for the aggressiveness and lethality of this disease. However, as slowly accumulating patient data converge on a number of signaling pathways that appear to be crucial in the establishment and development of ATC, genetically engineered mouse models based on such alterations have emerged in the past few years with the purpose of offering a physiological tool in the study of this malignancy. These mouse models of ATC are now allowing researchers a closer look at the mechanistic pathways responsible for aggressiveness and drug resistance observed in ATC, and they will thus continue to be invaluable tools.

Two signaling pathways have been shown to be commonly deregulated in ATC: the PI3K and the MAPK cascades. Differentiated thyroid tumors also show deregulations in these signaling axes; however, additional mutations, such as the mutation or deletion of *TRP53*, are characteristically found in ATC suggesting a stepwise progression from DTC to ATC [40, 32]. Thus, much effort has been put into investigating the role of these pathways in thyroid anaplastic tumors, as they represent the driving force behind their establishment and progression and a key to finding targets that will allow for the development of effective therapeutic approaches.

RET/PTC

Although this review focuses on ATC mouse models carrying mutations or deletions affecting the PI3K and MAPK pathways, it is also important to mention that the earliest model to present the characteristic ATC phenotype was generated through thyroid-specific expression of the RET/PTC1 oncogene in combination with a homozygous deletion of *Trp53* [30]. These *RET/PTC1⁺;Trp53^{-/-}* mice developed papillary thyroid tumors, which showed signs of progression to ATC, including solid areas composed of pleomorphic spindle, polyhedral, or multinucleated giant cells. The incidence of ATC was higher in mice with a homozygous deletion of *p53* in comparison to heterozygous mice, and this increased with age. At 14-17 weeks, 30% of all *RET/PTC1⁺;Trp53^{-/-}* mice presented with ATC features, and a local invasion was frequently observed [30]. While this model might be useful in the study of ATC, some limitations are readily observed, one of them being the fact that all of the *RET/PTC1⁺;Trp53^{-/-}* mice had to be removed from the study by 17 weeks of age, due to extrathyroidal tumors caused by the systemic *Trp53* deletion. In fact, while it is known that deficiency of *Trp53* is not enough to drive tumorigenesis in the thyroid [2], it is sufficient to initiate tumors in other tissues [30, 2]. A second major disadvantage of this model is that while the *RET/PTC1* translocation is one of the most common mutations associated with PTC, there is lack of evidence showing that human papillary thyroid tumors with RET/PTC1 can progress to ATC [54, 58].

RAS-MAPK Signaling Pathway

The mitogen-activated protein kinase (MAPK) pathway encompasses different signaling systems that are responsible for cell growth, proliferation, differentiation, migration and apoptosis. One of these systems is the *RAS*→*RAF*→*MEK*→*ERK1/2* cascade, which is deregulated in one third of all human cancers [34, 11]. Additionally, 70% of thyroid cancers

harbor mutations that can activate the MAPK pathway [11, 31, 29]. RAS GTPases act as switches, turning on signaling cascades upon stimulation of receptors at the membrane. Common point mutations in these proteins, which often occur in codons 12, 13 or 61, block GTP hydrolysis, thus rendering the protein constitutively active [11]. There are three human *RAS* genes (*H-RAS*, *N-RAS*, and *K-RAS*), and activating point mutations in these genes have been found in many different types of cancer. A study on the significance of *RAS* mutations in 107 thyroid cancer patients showed that 74.3% patients with *RAS*-mutated tumors died as a result of their disease in comparison to 31.9% patients with tumors lacking these mutations [15]. *RAS* mutations (most frequently *N-RAS*) are found in ATC with a variable frequency ranging from 8-60% depending on the sample series [40, 17, 15]. RAF proteins are direct effectors of RAS, and B-RAF is the strongest activator of MEK and the most commonly mutated of the RAF proteins [11]. *B-RAF* has been reported to be mutated in approximately 7% of all cancers; nevertheless, mutations in *B-RAF* are found in approximately 50% of papillary thyroid tumors and 25-44% of ATC cases [11, 48, 50, 23]. The most common B-RAF mutation, V600E, involves a change in the activation loop which induces constitutive activation [11, 40]. Both *RAS* and *B-RAF* activating mutations have been shown to confer drug resistance to thyroid carcinoma cell lines [44]. Therefore, mouse models of ATC based on alterations of key proteins of the *RAS-MAPK* signaling pathway allow a clinically and physiologically appropriate study of the role of this pathway in the progression of human thyroid cancer and therapy resistance.

RAS Models

A recently described mouse strain that carries a deletion of *Trp53* and expresses *Kras*^{G12D} specifically in the thyroid through the TPO-cre transgene models tumor progression from PTC to poorly differentiated thyroid cancer (PDTC) and ATC. These mice develop tumors starting at 5 months of age with full penetrance. Notably, 40% of tumors developing in these mice contain areas of ATC, characterized by a pleomorphic spindle cell pattern and a high mitotic index, with atypical mitosis and large areas of tumor necrosis. [8, 60, 7] 28% of mutants had lung metastases, an important feature considering that the most common sites for metastasis in ATC are the lungs [4]. Expression of epithelial-to-mesenchymal transition (EMT) markers and loss of thyroid differentiation markers such as *Tpo*, *Tg*, and *Slc5a* is observed, paralleling the increment of the ATC component [8]. All these features are hallmarks of human ATC [38]. Interestingly, these tumors show no immunoreactivity for activated AKT, suggesting that activated RAS is not functionally linked to the PI3K pathway in this model.

MEK inhibition in cell lines established from these tumors causes growth inhibition brought by an arrest in the G1 phase, however cell death is not observed. Further investigation has shown that cell death resistance is associated with overexpression of *Bcl2a1* and *Mcl1*, two of the anti-apoptotic members of the Bcl2 family of proteins [8]. De-regulation in the expression of the anti-apoptotic members of this family has been reported in many different types of cancer [63]. *BCL2A1* is involved in resistance to BRAF inhibition in melanoma [21], and *MCL1* has been reported to be expressed in undifferentiated thyroid cancer [5]. In fact, analysis of a large expression-profiling dataset derived from human thyroid cancers [16] reveals that overexpression of *MCL1* and *BCL2A1* is common among more aggressive

forms of thyroid cancer [8, 16]. Accordingly, treatment of *Kras*^{G12D};*Trp53*^{-/-} cell lines with a pan-inhibitor of the anti-apoptotic members of the Bcl2 family, Obatoclax, shows a drastic increase in cell death and synergize with the MEK inhibitor GSK1120212 both *in vitro* and *in vivo*, in syngeneic allograft models [8]. Whether this inhibitor cocktail has efficacy on autochthonous tumors in *Kras*^{G12D};*Trp53*^{-/-} mice remains to be seen [21, 5]. Thus, this model lends itself for further investigation of cell death resistance pathways, a critical area in understanding ATC drug resistance.

Mice expressing a mutant thyroid hormone receptor β (Thrb^{PV/PV}), spontaneously develop well-differentiated follicular thyroid cancer [57]. When this mutation is combined with thyroid-specific activation of Kras, compound mutants develop aggressive carcinomas with frequent presence (>60%) of areas of anaplasia [64].

Furthermore, lung metastases are observed in over 30% of mice. Interestingly, tumors with anaplastic foci display increased levels of MYC and reduced levels of the pro-apoptotic molecule, BIM, suggesting a direct link between the aggressive and drug-resistance features of ATC and the expression levels of these markers.

BRAF^{V600E} Models

Mice with a thyroid-specific BRAF^{V600E} mutation mediated by TPO-Cre^{ER} upon tamoxifen treatment develops PTC 12 weeks post induction [35]. When this model is crossed to an inducible *Trp53* mutant allele, the resulting TPO-Cre^{ER};*Braf*^{CA/+};*Trp53*^{R270H/+} model develops PTC that progresses to ATC in 50% of the cases. Furthermore, homozygous deletion of *Trp53* in these mice or the presence of the R270H point mutant combined with the loss of the wt *Trp53* allele accelerates the appearance of ATC and decreases the median survival in these mice [35]. The 6-month latency after induction of the BRAF mutation and loss of p53 suggests not only that additional oncogenic events need to take place before the appearance of ATC, but also that this model will be of use for studying the transition from a well differentiated tumor to a fully de-differentiated neoplasm. These tumors show ATC characteristic features such as solid growth, spindle cell morphology, and pleomorphic giant cell pattern. Furthermore, 19% of these animals develop lung micrometastases [35, 4]. Contrary to other BRAF^{V600E} thyroid cancer models showing highly up regulated TSH, this model presents a modest elevation of TSH in comparison to control mice. Notably, TSH suppression mediated by T4 supplementation does not alter the overall tumor phenotype, suggesting that TSH signaling does not contribute to the phenotype. This is important because elevated levels of TSH are not observed in human thyroid cancer [2] [51].

Gene expression profiling of these ATCs revealed signatures of proliferation and EMT in comparison to the PTCs, which corresponds to what is also observed in human ATC. Further interrogation of the signaling mechanisms involved in the transition from PTC to ATC in these mice showed increased levels of phospho-Akt(S473) and phospho-S6(S235/236) only in the ATC compartments. These findings suggest that the PI3K and mTOR signaling might become activated during progression to ATC in this model; and this is a common event observed in human ATC [35, 32].

Vemurafenib/PLX4032 is a potent BRAF inhibitor with selectivity against the wild-type BRAF, BRAF^{V600E}, and c-RAF-1. This drug was approved by the FDA in 2011 for the treatment of advanced metastatic melanoma [43]. The particular case of a 51-year old man with BRAF-mutated ATC who responded successfully to vemurafenib treatment has been reported [52]. This finding makes a case for the consideration of vemurafenib in ATC treatment in patients who test positive for the BRAF^{V600E} mutation[52]. The efficacy of this BRAF inhibitor was tested on the TPO-Cre^{ER};Braf^{CA/+};Trp53^{R270H/+} model. Upon confirmation of the presence of ATC, mice were treated with PLX4720, a more bioavailable sister compound to vemurafenib. The treated group showed a statistically significant overall survival. However, upon closer examination, this was due to a decrease in the PTC but not in the ATC component size. Furthermore IHC showed that both treated and control tumors were positive for phospho-Erk staining, suggesting incomplete MAPK pathway inhibition, and for pAKT [35]. Since phase I clinical trials for vemurafenib it has been known that an incomplete inhibition of the MAPK pathway is achieved even at the maximum tolerated doses [14]. Furthermore, the ability of tumor cells to activate compensatory signaling pathways or mutate Ras and re-activate the MAPK pathway has also been heavily reported. Thus, combination treatments including the use of a MEK inhibitor together with a BRAF inhibitor might be one strategy to completely block this oncogenic pathway [46, 22]. In the TPO-Cre^{ER};Braf^{CA/+};Trp53^{R270H/+} model a combination treatment of PLX4720 and PD0325901, a MEK inhibitor, showed a dramatic tumor suppressive effect, where 3 out of 4 ATC-bearing animals showed complete regression [35]. Since the combination of MAPK and PI3K signaling inhibition was not investigated in this model, this remains a key opportunity for future studies[35].

An additional Braf^{V600E}-based mouse model was developed with a thyroid-specific expression of Braf^{V600E} along with the expression of a constitutively active *Pik3ca*, encoding the p110 α catalytic subunit of PI3K (*Pik3ca*^{Lat}). Tissue-specific recombination was achieved through a TG-Cre^{ER} transgene. By 8.5 months of age, 71% of TG-Cre^{ER};Braf^{CA/+};Pik3ca^{Lat/+} mice develop palpable tumors, become severely sick, and require euthanasia. These tumors present aggressive and invasive PTC morphology. Moreover 80% of them show presence of polygonal giant cells and/or spindle cell pattern, which is characteristic of human ATC. These features are never observed in single mutants. Additionally, areas of ATC within these tumors show an EMT phenotype with loss of E-cadherin and gain of Vimentin, along with the loss of expression of TTF-1 [9], similar to human ATC [13, 38]. In order to further characterize the collaboration between an activating mutation in BRAF and the PI3K signaling pathway, a similar model of ATC was generated substituting the mutation in PI3K for a homozygous deletion of *Pten*. These TG-Cre^{ER};Braf^{CA/+};Pten^{-/-} mice develop ATC between 3.5 and 8 months after tamoxifen treatment. The latency period in these mice is shorter than in the TG-Cre^{ER};Braf^{CA/+};Pik3ca^{Lat/+} model [9]. It is important to note that while the activating mutation in *Pik3ca* alone does not have an effect in the mouse thyroid, the deletion of *Pten* causes hyperplasia and FTC [3, 62], suggesting either that Pten function can still restrain constitutively active PI3K signaling, or that Pten exerts PI3K-independent tumor suppressive functions. A more mechanistic characterization of the TG-Cre^{ER};Braf^{CA/+};Pik3ca^{Lat/+} and TG-Cre^{ER};Braf^{CA/+};Pten^{-/-} models is needed to study the

interplay of these pathways in ATC, validating and extending *in vivo* the lessons learned from human ATC cells lines.

PI3K Signaling Pathway

The PI3K signaling pathway is known to play a crucial role in glucose uptake, cell growth and proliferation, cell adhesion and motility, and survival [61, 41]. Activation of receptor tyrosine kinases (RTK) at the membrane by external factors activate PI3K, which then phosphorylates phosphatidylinositol-4,5-bisphosphate to produce phosphatidylinositol-3,4,5-trisphosphate (PIP3). PIP3 allows AKT to be localized to the membrane where it becomes activated by PDK1. Once activated, AKT is able to target downstream effectors that will promote a plethora of biological effects. PTEN is a negative regulator of this signaling pathway, since it dephosphorylates PIP3 and terminates the PI3K-AKT signal [61]. Activating mutations in the p110 α catalytic subunit of PI3K have been found in 25-40% of cancers. PIK3CA is mutated in 15-25% of all ATC cases, and its amplification is found in 40% of ATC. Furthermore, loss of function alterations in PTEN are found in 4-16% of ATC [48]. RAS is known to activate the PI3K signaling pathway and the crosstalk between these two systems has been well documented. In fact, 81.3% of ATC have been found to carry genetic alterations that could activate both pathways [32]. Recent studies have shown that blocking these two essential pathways in aggressive thyroid cancer cells allow for their sensitization to novel targeted therapies and traditional chemotherapeutic compounds [37, 20].

Pten Loss Models

The *Pten*^{-/-};*Trp53*^{-/-} mouse model of ATC, driven by the TPO-Cre transgene, develops anaplastic thyroid tumors after 8-10 months of age. These tumors cause severe tracheal compression and invade into adjacent tissues. The presence of remnants of well differentiated follicular thyroid carcinoma intermixed with ATC and the fact that glands from younger mice only show the presence well differentiated tumors suggest a progression mechanism from FTC to ATC. The ATC component of these tumors presents spindle cell morphology, giant osteoclast-like cells, and areas of osseous metaplasia (Figure 1). 28% of these mice show metastases in the lungs; less often metastases are also observed in the liver [2, 4]. Anaplastic tumors show loss of thyroid differentiation markers such as *Tpo*, *Tg*, and *Slc5a5* and the expression of EMT markers such as Vimentin, in accordance with human ATC [2, 38, 55]. Aneuploidy is an additional characteristic in common between these murine tumors and human ATC [2, 28]. Interestingly, IHC analysis reveals that the levels of activated Akt are higher in the well-differentiated areas of the tumors in comparison with the anaplastic component. The relevance and consequences of this finding, which is in keeping with similar data obtained in human ATCs [33], remain to be explored. Nevertheless, cell lines derived from these mice show sensitivity to AKT inhibition.

Genome-wide expression profiling of tumors from *Pten*^{-/-};*Trp53*^{-/-} mice revealed a unique signature correlated with the development of ATC. Murine ATCs showed higher expression level of genes involved in metabolic remodeling, cell motility and invasiveness, leukocyte recruitment, and EMT. Notably, cross-comparison of these findings to gene expression

datasets from human ATCs showed a strong similarity between the mouse and human ATC profiles [2]. Commonalities between the two systems are likely to identify promising therapeutic targets, as exemplified by the upregulation of PLK1 [53]. Human ATCs display a strong uptake of glucose, suggesting a dependence on glycolysis for survival, a characteristic of some tumors that is defined as the Warburg effect [45]. In accordance to this, ATCs in *Pten*^{-/-};*Trp53*^{-/-} mice show a higher uptake of glucose in comparison to control mice, but also upregulate genes involved in the establishment of the Warburg effect such as *Hif1a*, *Hexokinase 2*, and *Pyruvate Kinase M2* and display a dramatic increase of lactate content. Most importantly, cell lines derived from these tumors were effectively inhibited by glycolytic inhibitors, which showed a synergistic effect when combined with doxorubicin, a drug currently used in the treatment of human ATC [2]. A major strength of this model lies in its thoroughly characterized morphological and physiological similarities to the human counterpart. This slow progression makes this model suitable for the study of the transition between well-differentiated thyroid cancer and ATC. On the other hand, the unpredictability of the time of tumor dedifferentiation is a limitation for preclinical studies. The development of several cell lines derived from these tumors, however, allows the establishment and preclinical use of syngeneic allograft models, partially addressing this issue [53].

Another mouse model presenting areas of ATC was generated by the simultaneous deletion of *Pten* and the expression of a dominant negative mutation of the thyroid hormone receptor- β . These *Thrb*^{PV/PV}*Pten*^{+/-} mice develop tumors with characteristics of FTC, which progress to display undifferentiated nests in 50% of mice older than 7 months, and metastasize to the lungs in about 80% of cases [19]. When *Thrb*^{PV/PV}*Pten*^{+/-} mice were fed a high fat diet (HFD) to induce obesity, 62% of them developed anaplasia by 21 weeks of age, in comparison to 24% in mice fed a low fat diet (LFD). Histopathological analysis of tumors developed by the HFD group showed loss of differentiation and the presence of spindle cell anaplasia.

Further analysis of these tumors also showed they stained more positively for Ki-67 than tumors in the LFD group [26, 41]. Furthermore, TSH levels of HFD and LFD mice did not show any differences, a characteristic consistent with the lack of increased TSH in advanced human thyroid cancer [7, 26]. Mechanistically, it was suggested that tumor progression depended on increased levels of leptin, an adipocyte-derived hormone upregulated in obesity, and that leptin acts through the JAK2-STAT3 signaling pathway, which is critical in cell survival and proliferation [26, 6, 42]. This mouse model presented with high levels of leptin upon HFD, and displayed upregulation of the JAK2-STAT3 pathway comparison to LFD controls. [26] However, the fact that the JAK2-STAT3 pathway is also activated by several factors other than leptin, calls for additional in depth analysis of this model [26, 56, 12, 24]. The limitations of this strain as an ATC model lie principally in the degree of anaplasia observed. Even when the original *Thrb*^{PV/PV} presented anaplasia in 35% of cases, this feature is restricted to small patches within the tumor [57]. While the concomitant deletion of one *Pten* allele accelerates tumorigenesis and the HFD make the anaplasia appear more readily, the level of dedifferentiation was still very limited [26, 47, 18, 25].

Conclusions

The rarity and aggressiveness of human ATC have been insurmountable barriers for research efforts focused on understanding the mechanisms of ATC development and designing rationale therapeutic approaches. The recent generation of different ATC mouse models encompassing most of the spectrum of ATC-associated mutations has opened new doors in the study of this malignancy. These models represent new tools that are helping understand the mechanisms involved in tumor progression and drug resistance. Particular enthusiasm is generated by models in which recombination can be induced postnatally, bypassing the proliferative wave that expands the thyrocytes pool between thyroid specification and 3-4 weeks after birth. A more detailed characterization of some of these models is still needed; for example, no data are available on the crosstalk between ATC cells and their microenvironment, and on the role immune cells infiltrating these tumors have in sustaining or countering tumor growth and spread. Nevertheless, expression profiling studies have already unveiled possible roles for specific signaling pathways in the establishment and progression of ATC. Future research will have to exploit these models to design and test innovative therapeutic modalities.

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Figure 1. Human (A) and mouse (B) ATC display similar morphological features, including the establishment of EMT, as shown by loss of E-cadherin (C) and expression of Vimentin (D).

Model	Cre	Tumor type	ATC Penetrance	Cellular features	Metastasis	Preclinical studies
<i>RET/PTC1⁺;Trp53^{-/-}</i>	N/A	PTC/ATC	60% at 11-14 weeks. Difficult to determine due to extra-thyroidal tumors	Pleomorphic spindle, polyhedral, or multinucleated giant cells. Mitotic figures	Only 1 case found (lungs)	N/A
<i>Kras^{G12D};Trp53^{-/-}</i>	TPO-Cre	PTC/PDTC/ATC	40%	Pleomorphic spindle or epithelioid cells	28% to the lungs	Inhibition of MEK by GSK1120212 and of anti-apoptotic Bcl2 family members by Obatoclax in allografts from tumor-derived cell lines
<i>Thrb^{flV/pV};Kras^{G12D}</i>	TPO-Cre	FTC/ATC	63% between 2 and 5 months	Spindle cell anaplasia	31% between 2 and 5 months	N/A
<i>Braf^{C4A/-};Trp53^{R270H/+}</i>	TPO-Cre ^{ER}	PTC/PDTC/ATC	50% PDTC or ATC	Tall cell morphology with focal necrosis. Spindle cells and pleomorphic giant cell pattern	19% to the lungs	Inhibition of BRAF by PLX4720 and of MEK by PD0325901
<i>Braf^{C4A/-};Plk3^{cd^lad/+}</i>	TG-Cre ^{ER}	PTC/ATC	80%	Polygonal giant cells and/or spindle cell pattern	Not reported	N/A
<i>Pten^{-/-};Trp53^{-/-}</i>	TPO-Cre	FTC/ATC	90%	Spindle cell morphology, giant, cells, osteoclast-like, multinucleated cells, and areas of osseous metaplasia	28% to the lungs Sporadic to the liver	Inhibition of PLK1 by GSK461364A in allografts from tumor-derived cell lines
<i>Thrb^{flV/pV};Pten^{+/-}</i>	N/A	FTC/ATC	50% after 7 months 62% after 21 weeks HFD	Spindle cell anaplasia	80% to the lungs	N/A