Mitochondrial Metabolism as a Treatment Target in Anaplastic Thyroid Cancer

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Anaplastic thyroid cancer (ATC) is one of the most aggressive human cancers. Key signal transduction pathways that regulate mitochondrial metabolism are frequently altered in ATC. Our goal was to determine the mitochondrial metabolic phenotype of ATC by studying markers of mitochondrial metabolism, specifically monocarboxylate transporter 1 (MCT1) and translocase of the outer mitochondrial membrane member 20 (TOMM20). Staining patterns of MCT1 and TOMM20 in 35 human thyroid samples (15 ATC, 12 papillary thyroid cancer [PTC], and eight non-cancerous thyroid) and nine ATC mouse orthotopic xenografts were assessed by visual and Aperio digital scoring. Staining patterns of areas involved with cancer versus areas with no evidence of cancer were evaluated independently where available. MCT1 is highly expressed in human anaplastic thyroid cancer when compared to both non-cancerous thyroid tissues and papillary thyroid cancers ($P < .001$ for both). TOMM20 is also highly expressed in both ATC and PTC compared to non-cancerous thyroid tissue ($P < .01$ for both). High MCT1 and TOMM20 expression is also found in ATC mouse xenograft tumors compared to non-cancerous thyroid tissue ($P < .001$). These xenograft tumors have high ^{13}C- pyruvate uptake. ATC has metabolic features that distinguish it from PTC and non-cancerous thyroid tissue, including high expression of MCT1 and TOMM20. PTC has low expression of MCT1 and non-cancerous thyroid tissue has low expression of both MCT1 and TOMM20. This work suggests that MCT1 blockade may specifically target ATC cells presenting an opportunity for a new drug target.

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Thyroid cancer is the most common endocrine malignancy in the United States, but anaplastic thyroid cancer (ATC) constitutes only 1% of these cases.[1] This histology confers a particularly poor prognosis with a median survival of only 3–5 months.[2] Patients usually succumb to local invasion, as ATC metastasizes in only 10%–20% of cases.[3] These tumors are generally chemo-resistant, so the mainstays of treatment are surgical resection and radiation therapy. The pathogenesis of ATC is poorly understood, although key regulators of cellular metabolism such as *PI3K*, *PTEN*, *RAS*, and *TP53* are frequently mutated. We hypothesized that altered mitochondrial metabolism is a feature of ATC. To test this we compared expression of two markers of mitochondrial metabolism, monocarboxylate transporter 1 (MCT1) and translocase of the outer mitochondrial membrane member 20 (TOMM20) in ATC to that observed in noncancerous thyroid tissue and papillary thyroid cancer (PTC).

MCT1 is a member of the *SLC16A* gene series and is a proton-linked bidirectional transporter of monocarboxylic acids, specifically L-lactate, pyruvate, acetoacetate, propionate, and D,L-β-hydroxybutyrate.[4] Expression of MCT1 at the cell surface allows for either the import or export of monocarboxylates.[5] MCT1 is the most ubiquitous of the four known monocarboxylate transporters (MCTs) and is expressed in red muscle fibers, myocardiocytes, and hepatocytes.[6–8] All of these cell types have high rates of oxidative phosphorylation (OXPHOS) facilitated by the presence of MCT1 as the monocarboxylates taken up by MCT1 are incorporated into the mitochondrial tricarboxylic acid (TCA) cycle and the ones MCT1 exports such as β-hydroxybutyrate and acetoacetate are generated in the mitochondria.[9,10]

MCT1 expression is under the control of *TP53* and *MYC*.[11,12] Disruption of MCT1 function can cause an increase in intracellular lactate concentration with downstream effects of reduced glucose transport, reduced levels of adenosine triphosphate (ATP), NADPH, and glutathione. As a consequence of the reduced levels of glutathione, cells show increased levels of hydrogen peroxide, mitochondrial damage, and apoptosis.[12] High MCT1 expression has been found in numerous human cancer cells compared to non-cancer cells.[13–16] Expression levels of MCT1 are prognostic in renal cell cancer and non-small cell lung cancer.[17,18]

The role of MCT1 in normal thyroid metabolism has been investigated in an in vitro model with cultured rat FRTL-5 cells where MCT1 was found to be expressed. Thyroid-stimulating hormone (TSH) was noted to upregulate MCT1 expression in this model system.[19] To our knowledge there are no studies which have examined the expression of MCT1 in human thyroid tissue, including ATC.

Expression of the translocase of the outer mitochondrial membrane member 20 (TOMM20) is a marker of mitochondrial oxidative phosphorylation (OXPHOS) metabolism.[20–22] Increased uptake of nuclear encoded mitochondrial OXPHOS proteins is a mechanism by which cells adapt to increased energy demand.[20] Only 13 of the OXPHOS subunit proteins are encoded by the mitochondrial genome and hence the majority of the subunits are encoded in the nucleus and must be imported to the mitochondria through the translocase of the outer membrane (TOM) complex.[23] TOMM20 is the receptor subunit of the mitochondrial import pore.[24] No publications to our knowledge have studied the expression of TOMM20 in either normal thyroid tissue or in thyroid cancers. TSH increases TOMM20 expression in cardiac myocytes.[25] TOMM20 also has been investigated in cancer cell metabolism and in gastric cancer has been shown to be a negative prognostic marker.[26] In head and neck cancer, high TOMM20 expression is found in highly proliferative (Ki-67–positive) cancer cells.[21]

In summary, MCT1 is a bidirectional transporter of monocarboxylates such as lactate, pyruvate, β-hydroxybutyrate, and acetoacetate. MCT1-mediated uptake of monocarboxylates increases mitochondrial metabolism as they serve as substrates for the TCA cycle and OXPHOS. Conversely the export of monocarboxylates generated via mitochondrial metabolism via MCT1 protects cells from catastrophic oxidative stress. TOMM20 is the main subunit of the import pore for proteins to enter the mitochondria and it allows mitochondrial localization of nuclear encoded OXPHOS subunits. Expression of TOMM20 correlates with mitochondrial mass and OXPHOS function. In this study, we have partially characterized the metabolic profile of anaplastic thyroid cancer by analyzing expression of MCT1 and TOMM20 in human specimens and mouse orthotopic xenograft tumors.

MATERIAL AND METHODS

Subjects

The protocol for this study was approved by the Institutional Review Board at Thomas Jefferson University. Tissue specimens of anaplastic thyroid cancer were obtained from 15 subjects at Thomas Jefferson University Hospital. Patient data were collected including age, sex, staging, tumor morphology, treatment, and recurrence.

Samples and Immunohistochemistry

A total of 15 human samples of anaplastic thyroid carcinoma were studied to evaluate markers associated with metabolism in the cancer cells of our tumor samples. Eight noncancerous thyroid specimens including five nodular goiters, three hyperplastic nodules, and one benign biopsy were used as normal controls. Twelve specimens of papillary thyroid cancer also were used for comparison. All human cases studied were from 2010 through 2014. For all cases, all available surgical resection or biopsy materials were reviewed to confirm the diagnosis. Samples consisted of a minimum of a core biopsy. No needle aspirate specimens were considered. Nine ATC xenografts were studied by MCT1 and TOMM20 immunohistochemistry.

Samples were stained by immunohistochemistry for MCT1 and TOMM20. All cancer present on a slide and its dominant staining pattern were considered when determining the percent of immunohistochemistry-positive cancer cells in a sample. Each case was reviewed independently by two pathologists who performed the evaluation blinded to clinical outcome.

An empirically derived scaling system was created based on evident staining patterns in the samples. An MCT1 staining score of low or high was given if $<30\%$ or $\geq 30\%$ of cells stained strongly on the plasma membrane. TOMM20 was considered high when at least 70% of cells demonstrated strong, diffuse cytoplasmic staining. All other cases were considered low.

Quantitative analysis of TOMM20 was also performed employing digital pathology with Aperio software as previously described.[27] Briefly, tissue sections were scanned on a ScanScope XT with an average scan time of 120 seconds (compression quality 70). Images were analyzed using the Color Deconvolution and the Colocalization Aperio Image Analysis tool. Areas of staining were color separated from hematoxylin counter-stained sections and the intensity of the staining was measured on a continuous 0 (bright white) to 255 (black) scale. For each case three representative areas of the tumor were analyzed.

Human tissues for analysis were fixed in neutral buffered formalin and then embedded in paraffin. Sections (4 μm) were dewaxed, rehydrated through graded ethanols, and antigen retrieval was performed in 10 mmol/L citrate buffer, pH 6.0, for 10 min using a pressure cooker. The sections were cooled, blocked with 3% hydrogen peroxide and then for endogenous biotin using the Dako Cytomation Biotin Blocking Sytem (Dako). Sections were next incubated with 10% goat serum for 30 minutes, followed by primary antibodies overnight at 4°C (Santa Cruz Biotechnology, Santa Cruz, CA; MCT1 H-1, sc-365501 and TOMM20 F-10, sc-17764). Primary antibody binding was detected with a biotinylated species-specific secondary antibody (Vector Labs) followed by a streptavidin-horseradish peroxidase conjugate (Dako). Immunoreactivity was revealed with 3,3′-diaminobenzidine (Dako). Sections were counterstained with hematoxylin.

Orthotopic Xenograft Model

In addition to human samples, orthotopic xenograft tumors of ATC were examined. Male athymic nude mice (8–12 weeks old) were purchased from the National Cancer Institute, maintained in a specific pathogen-free animal facility and fed with sterilized food and water in laminar flow cabinets. Animal procedures and handlings were in accordance with the protocol approved by the IACUC at University of Texas MD Anderson Cancer Center. Male athymic nude mice were injected with 2.5 x 10^5 U-HTH83 cells expressing a luciferase reporter into the right thyroid lobe as previously described.[28–30] The original U-HTH83 cell line was established from a 66-year-old man with a rapidly enlarging giant cell ATC.[31] Animals were killed and tumors were harvested. Sectioned materials were stained in an identical fashion to those of our patient samples, as detailed above.

Statistics

The expression of MCT1 and TOMM20 in human anaplastic thyroid specimens was compared to both non-malignant and PTC specimens considering the staining as a continuous variable using analysis of variance (ANOVA) and assuming equal variance in the data sets.

RESULTS

Baseline Patient Characteristics

Our patients included 15 individuals with a diagnosis of ATC treated at Thomas Jefferson University Hospital between the years of 2010 and 2014 as shown in Table 1. The female to male ratio was 3:2 and the median age was 75.1 years (range 51–84). The staging of these tumors was as follows: eight of the 15 patients had confirmed nodal involvement at the time of diagnosis, four patients did not have nodal involvement, and in three patients the lymph nodes were not assessed. Multiple histologic subtypes were represented with two of 15 with giant cell features, five with squamous differentiation, and three with spindle cells. Seven of the 15 tumors appeared to have arisen within the background of a papillary carcinoma, while one appeared to have arisen in a follicular carcinoma. Eight of the 15 patients underwent a surgical debulking procedure with lymph nodes addressed while the other seven patients were diagnosed with core biopsy only before proceeding on to radiation therapy. Two patients enrolled in hospice after their diagnostic biopsy and another patient declined further treatment after a total thyroidectomy and lymph node dissection. Of the remaining 12 patients, nine had multimodal therapy with chemotherapy and radiation and three underwent radiation alone. Chemotherapeutic regimens were highly varied and included docetaxel, paclitaxel, cisplatin, carboplatin, doxorubicin, sorafenib, and an ongoing clinical trial with pazopanib (Radiation Therapy Oncology Group [RTOG] 0912).

Survival Data

Of the 15 patients presented here, two were alive at the time of data analysis. Their survival time was calculated as of our defined cut-off date. When all patients, including those alive at the time of our data analysis, are included the median survival time was 188 days with a range from 15–447+ days. When only those who have succumbed to their disease are analyzed (13 of the 15 cases) the average survival is 161 days. Thus these cases appear to be

Table 1. Patient Characteristics, MCT1, and TOMM20 Expression by Immunohistochemistry.

Sample	TOMM20 Cancer	MCT1 Cancer	Sex	Age (yr)	T	N	M	Surgery	Treatment	Survival (days)
1	High	High	M	54	4b	1	0	Total, RND	Chemo/XRT	447*
2	High	High	M	83	4b	1b	0	Total, BLND	Chemo/XRT	208
3	High	High	F	83	4b	0	0	Bx	Chemo/XRT	304
4	High	High	F	73	4b	1b	0	L lobe, BLND	Chemo/XRT	205
5	High	High	F	84	4a	0	0	Bx	N	19
6	High	High	F	82	4b	X	1	Bx	N	15
7	Low	High	F	84	4b	X	1	Bx	XRT	46
8	High	Low	M	79	4b	1b	1	Total, PND, LND	Chemo/XRT	149
9	High	High	F	83	4b	X	1	Bx	XRT	163
10	High	Low	F	70	4b	1b	1	Total, BLND	Chemo/XRT	215
11	High	High	F	75	4b	1a	0	Total, BLND	Chemo/XRT	243
12	Low	High	M	61	4a	1b	0	Total, LND	N	115
13	High	High	F	79	4b	1b	0	Bx	Chemo/XRT	224
14	Low	High	M	84	4a	0	0	Bx	XRT	189
15	High	High	M	51	4a	0	0	Total, PND	Chemo/XRT	280*

* Alive as of September 1, 2015.
NOTE: T, N, M indicate tumor, node, and metastasis stage; Surgery: Total = total thyroidectomy; RND = right neck dissection; LND = left neck dissection; BLND = bilateral neck dissection; PND = paratracheal neck dissection; Bx = biopsy.

representative of anaplastic thyroid cancer with respect to survival statistics.

High Expression of MCT1 Is Seen in ATC Specimens Relative to Noncancerous and PTC Specimens

In the panel of 15 ATC specimens, 13 samples showed high expression of MCT1 (Table 1, Figure 1C). In contrast, PTC and noncancerous thyroid tissue failed to show this high level of expression (Figures 1A, B). In summary, 13 of 15 ATC had high expression of MCT1, none of eight normal samples had high expression ($P < .0001$) (Figure 1E). In the 12 samples of PTC, 10 had low levels of staining for MCT1 ($P < .001$) (Figure 1E). Of the two that stained for MCT1, one sample represented the contralateral lobe of a patient who had presented with anaplastic thyroid cancer (specimen no. 15 in Table 1). Thus, MCT1 protein expression is a distinguishing feature of anaplastic thyroid cancer compared to PTC and noncancerous thyroid samples. When the orthotopic xenograft mouse models also were assessed by immunohistochemistry, all samples tested demonstrated high expression of MCT1 in the tumor cells, reiterating this phenotype (Figure 1E, Table 2). Surrounding noncancerous mouse tissue did not stain for MCT1.

High TOMM20 Is Observed in ATC Specimens Relative to Noncancerous Thyroid Tissue

The functional mitochondrial mass of anaplastic thyroid cancer cells was assessed via TOMM20 expression. High expression of TOMM20, defined as >70% of the cells staining, was seen in 12 of the 15 cases of ATC (Table 1, Figure 2C). Low expression of TOMM20 was seen in all eight samples of noncancerous thyroid tissue (Figure 2A). Similar to the ATC specimens, PTC demonstrated high staining in contrast to the noncancerous thyroid tissue (Figure 2B). When TOMM20 expression by immunohistochemistry was assessed in the orthotopic xenograft model, the cancer cells in all nine samples stained in a pattern similar to the human ATC specimens (high staining of TOMM20 in tumor tissue and none in surrounding mouse tissue) again reiterating this metabolic signature (Figure 2E, Table 2).

We further investigated the staining of these samples using digital quantification. Comparisons were made between the carcinoma cells from patients with ATC and the noncancerous thyroid tissue in the same samples with the Aperio digital quantification system. This system is able to compare the overall intensity of stain per object (tumor cell) in a selected area. With a previously validated algorithm for TOMM20 analysis we were able to establish an average stain intensity of 164.06 in our anaplastic specimens versus 177.0 in the noncancerous controls. The higher numeric value in this system correlates to a lower stain intensity. Thus, there is significantly more TOMM20 staining in ATC compared to noncancerous thyrocytes ($P < .05$). To account for the higher numeric value correlating with a lower stain intensity, these were graphically represented as mathematical inverses (Figure 2D).

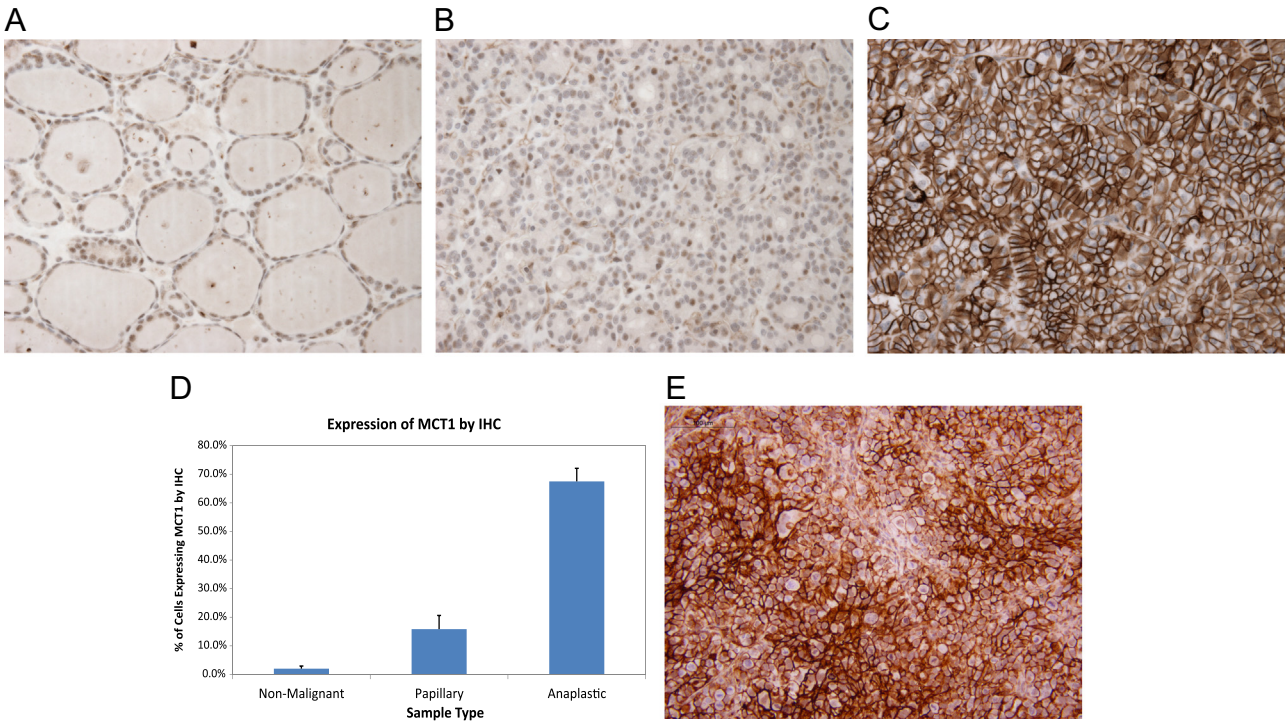

Figure 1. Representative photomicrographs showing MCT1 staining by immunohistochemistry in patient samples representing: (A) noncancerous, normal thyroid tissue, (B) papillary thyroid carcinoma (PTC) and (C) human anaplastic thyroid carcinoma (ATC). (D) Quantification of the average number of cells expressing MCT1 by immunohistochemistry seen in normal human thyroid tissue as compared to PTC and ATC; error bars represent the standard error of the mean. (E) MCT1 staining of orthotopic xenograft model of ATC. All images are at 40x magnification.

To determine if this elevation of mitochondrial mass was also present in other thyroid malignancies, we compared ATC to PTC. Twelve PTC cases were compared using the same TOMM20 Aperio algorithm to the ATC cases as described above. There was no statistically significant difference in average intensity between PTC (intensity of 160.2) compared to ATC (intensity 164.06) ($P > .05$) (Figure 2D). However, as previously described, TOMM20 intensity is greater in ATC than in the noncancerous samples ($P < .001$).

DISCUSSION

We demonstrate here that in human anaplastic thyroid cancer there is high expression of MCT1 and TOMM20 compared to noncancerous thyroid tissue. The high expression levels of these two markers of monocarboxylate transport and mitochondrial metabolism support our hypothesis that deranged cellular metabolism is a feature of ATC.

High MCT1 expression is a feature of ATC compared to normal thyroid tissue and PTC. We

Table 2. MCT1 and TOMM20 Expression by Immunohistochemistry in Mouse Xenograft Models

Specimen No.	TOMM20 Cancer	TOMM20 Non-cancer	MCT1 Cancer	MCT1 Non-cancer
1	High	Low	High	Low
2	High	Low	High	Low
3	High	Low	High	Low
4	High	Low	High	Low
5	High	Low	High	Low
6	High	Low	High	Low
7	High	Low	High	Low
8	High	Low	High	Low
9	High	Low	High	Low

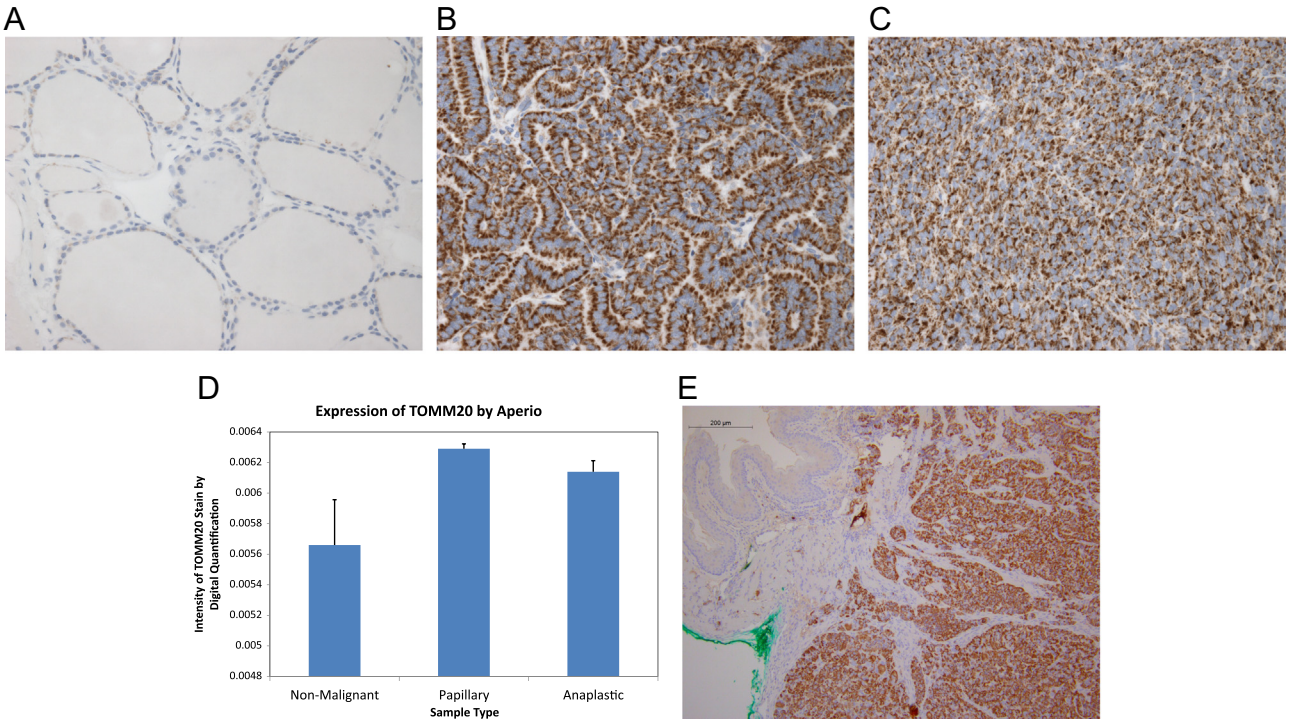

Figure 2. Representative photomicrographs showing TOMM20 staining by immunohistochemistry in patient samples representing: (A) noncancerous, normal thyroid tissue, (B) papillary thyroid carcinoma (PTC), and (C) human anaplastic thyroid carcinoma (ATC). All images are at 40x magnification. (D) Average stain intensity of TOMM20 by digital quantification of immunohistochemistry in normal human thyroid tissue as compared to PTC and ATC. The Y axis represents the inverse of the numeric output of the Aperio analysis system. Error bars represent the standard error of the mean. (E) TOMM20 staining of orthotopic xenograft model of ATC. Image at 20x magnification.

hypothesize that this is directly related to the high rate of OXPHOS being conducted in these cancerous cells, similar to other metabolically active cell types already known to express MCT1. MCT1's presence would enable import of pyruvate and lactate to fuel mitochondrial respiration,[32–35] giving them a significant growth advantage.

While MCT1 is highly expressed in the ATC samples, the PTC samples rarely showed high expression. This contrast is reiterated in the clinical phenotypes of the two cancers. ATC shows high growth kinetics and a short overall survival while PTC is more indolent with a much better prognosis. The 5-year overall survival for ATC is 7% while for PTC it is over 90%. Well-differentiated thyroid carcinoma is thought to be the precursor lesion for the development of ATC. In future in vitro experiments it will be important to determine if high MCT1 is necessary or sufficient to induce the transformation of PTC into ATC.

Also of interest is the identification of the main monocarboxylates being transported by MCT1 in ATC and whether MCT1 is involved predominantly in their import or export in this disease. High uptake of ^{13}C- pyruvate in tumors has been noted in these ATC xenografts.[28,36] Uptake of ^{13}C-pyruvate in these xenografts is most likely mediated by MCT1 since this is the main importer of pyruvate into cells, but this will need to be confirmed experimentally.

High TOMM20 expression also is found in human ATC in contrast to low expression in normal thyrocytes. Like the expression of MCT1, TOMM20 expression is associated with high OXPHOS as it is a key component of the mitochondrial outer membrane (MOM) protein import system for nuclear encoded subunits of OXPHOS,[37] specifically, the import of precursor proteins containing mitochondrial targeting sequences.[22] It has been shown that TOMM20 RNA interference inhibits the MOM targeting and mitochondrial localization of nuclear encoded respiratory chain complexes, whereas TOMM20 overexpression has the opposite effects.[22] Future studies will need to discover the regulators of TOMM20 expression in ATC.

The involvement of mitochondrial metabolism shown in this paper is also supported by the genetic signature of ATC. Frequently found mutations in ATC include *TP53*, *H-*, *N-*, or *KRAS*, *BRAF* (V600E mutation), *PIK3CA*, *PIK3CB,* and *PTEN*.[38–40] Hence, the most frequent genetic alterations in ATC are in the RAS/RAF/ERK and the PI3K/PTEN pathway. Both of these signal transduction pathways are known metabolic modulators. *RAS* drives mitochondrial metabolism. Activation of *RAS* signaling increases

the mitochondrial TCA cycle.[41] This classic member of the MAPK pathway also plays a role in the PI3K/AKT pathway by binding directly to PI3K at the p110 subunit. Loss of wild-type *TP53* also increases mitochondrial metabolism[42] and in conjunction with activation of *RAS* increases OXPHOS.[43] *TP53* mutations also induce expression of MCT1.[11] In summary, the current literature on genetic alterations in ATC both supports the involvement of mitochondrial metabolism and specifically the involvement of MCT1. In the future it will be of interest to determine experimentally if they are the drivers of the ATC metabolic phenotype discovered in the current study.

Despite our growing knowledge of the genetic alterations in ATC, it has been difficult to target these mutations to improve outcomes in this disease. By studying the metabolic characteristics of ATC and finding differences with noncancerous tissues and precursor lesions such as PTC we can develop and study drugs that preferentially target the cancer cells and spare normal counterparts. It already has been shown that inhibitors of glycolysis such as 2-deoxyglucose preferentially target ATC in animal models.[36] Currently, MCT1 inhibitors are being tested for the treatment of human malignancies and may be valuable for the treatment of ATC.[44] Also, mitochondrial inhibitors such as metformin may hold promise to treat ATC.[45] The two markers of mitochondrial metabolism assessed here, TOMM20 and MCT1, are highly expressed in ATC compared to normal thyrocytes and future work will determine if these are viable therapeutic targets.

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