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Expression of angiogenic switch, cachexia and inflammation factors at the crossroad in undifferentiated thyroid carcinoma with *BRAF*^{V600E}

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Abstract

Cachexia is the result of complex metabolic alterations which causes morbidity in patients with advanced cancers including undifferentiated (anaplastic) thyroid carcinoma (ATC). ATC is a lethal disease with limited therapeutic options and unclear etiology for cachexia. We hypothesize that the *BRAF*^{V600E} oncoprotein triggers microvascular endothelial cell tubule formation (*in vitro* angiogenesis) by means of factors which play a crucial role in angiogenic switch, inflammation/immune response and cachexia. We use human ATC cells and applied multiplex ELISA assay to screen for and measure angiogenic/cachectic and pro-inflammatory factors in the ATC-derived secretome. We find that vemurafenib anti-*BRAF*^{V600E} therapy significantly reduces secreted VEGFA, VEGFC and IL6 protein levels compared to vehicle-treated ATC cells. As a result, the secretome from vemurafenib-treated ATC cells inhibits microvascular endothelial cell-related *in*

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Authors' Contributions

Conception and design: CN

Writing of the manuscript: CN

Development of methodology: CN

Acquisition of data: CN, AH, NH, PMS

Analysis and interpretation of data (e.g. statistical analysis, biostatistics, computational analysis): CN, AH, PMS

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vitro angiogenesis. Furthermore, ATC clinical samples express VEGFA, VEGFC and IL6 proteins. Our results suggest that angiogenic/cachectic and pro-inflammatory/immune response factors could play a crucial role in *BRAF*^{V600E}-positive human ATC aggressiveness. Understanding the extent to which microenvironment-associated angiogenic factors participate in cachexia and cancer metabolism in advanced thyroid cancers will reveal new biomarkers and foster novel therapeutic approaches.

Keywords

anaplastic thyroid cancer; cachexia; cytokines; angiogenesis; metastasis; inflammation; microenvironment; *BRAF*^{V600E}

Introduction

Anaplastic (undifferentiated) thyroid carcinoma (ATC) is an advanced thyroid cancer and one of the most aggressive malignancies, with a median survival rate of about 3–5 months [1; 2; 3]. ATC frequently manifests as a rapidly expanding neck mass [4], and more than 80% of ATC patients present with primary tumors exhibiting extensive infiltration of regional tissue structures and prominent vascular invasion with local and distant metastasis [5]. As ATC is resistant to standard treatment modalities, including chemotherapy, external beam radiation, and radioiodine therapy, reimagined therapeutic paradigms are needed [6] [7] [8] [9] [10]. The *BRAF*^{V600E} mutation is the most prevalent genetic alteration in PTC (frequency: about 60%) and may be associated with tumor progression of PTC to ATC (frequency: about 15–44%) [11; 12; 13; 14; 15; 16; 17; 18]. Any rational targeted therapy would require attention to *BRAF*^{V600E}-positive papillary thyroid carcinoma (PTC) or ATC [14; 19; 20]. Vemurafenib is the first orally available selective inhibitor of *BRAF*^{V600E} approved by the FDA (Food and Drug Administration) for the treatment of *BRAF*^{V600E}-melanoma. In advanced stage malignant diseases, the poorer survival of the patient is associated with cachexia, a debilitating syndrome of anorexia, progressive weight loss and depletion of adipose tissue and muscle mass. Cachexia is a frequent manifestations of malignancy and is estimated to be the immediate cause of death in 20–40% of cancer patients [21] [22]. Elements of key intracellular signaling cascades have been postulated to play important roles in the onset of cachexia in advanced human cancers. In particular, angiogenic and pro-inflammatory/immune response cytokines such as TNF (tumor necrosis factor)-alpha and IL (interleukin)-6, as well as other factors such as proteolysis inducing factor (PIF) [3] are involved in pro-cachectic mechanisms. Recently, involvement of tumor-derived parathyroidhormone-related protein (PTHrP) has been also reported to facilitate transcriptional regulation of genes fundamental for thermogenesis and adipose tissue homeostasis [23]. Hormones such as leptin or neuropeptides (e.g. neuropeptide Y), melanocortin, melanin-concentrating hormone and orexin have also been associated with the pathogenesis of cachexia [5], as have increased levels of thyroid hormones, including total T3 and free T3 [24]. In male patients with advanced cancers, low levels of total testosterone have been linked to cachexia and decreased survival [25]. Overall, cachexia and angiogenesis-associated pathways may be functionally interconnected and trigger cancer progression. We have previously shown that *BRAF*^{V600E}-positive human 8505c ATC

orthotopic mice began to lose weight by day 28 after tumor implantation, developed progressive cachexia and required sacrifice by day 35 [26]. At 5 weeks after tumor implantation, we also found higher CD31 immunoexpression (marker of microvascular density/angiogenesis) in the orthotopic sh-control 8505c ATC as compared to the orthotopic 8505c ATC with knockdown of BRAF^{V600E} [12]. Importantly, ATC orthotopic mice treated with PLX4720 anti-BRAF^{V600E} targeted therapy maintained their weight and showed no signs of cachexia. However, angiogenic and cachectic factors associated to BRAF^{V600E} and involved in ATC disease are still poorly characterized. Here, we have investigated angiogenic/cachectic and pro-inflammatory factors using a multiplex ELISA (Enzyme-linked immunosorbent) in vehicle-treated or vemurafenib-treated secretome derived from ATC cells with BRAF^{V600E}. We also studied *in vitro* angiogenesis by using vehicle-treated or vemurafenib-treated secretome derived from human ATC cells. Remarkably, BRAF^{V600E}-ATC samples showed increased angiogenesis compared to BRAF^{WT}-ATC samples. Our results suggest that metastatic BRAF^{WT/V600E}-ATC cells *in vitro* secreted significantly higher protein levels of VEGFA, VEGFC and IL6 which are known to be involved in the angiogenic switch (generally referred as tumor begins to overexpress angiogenic molecules), cachexia and inflammation. More importantly, their protein levels and *in vitro* angiogenesis (tubule formation) were greatly down-regulated in the presence of vemurafenib-treated secretome derived from BRAF^{V600E}-ATC cells.

Material and Methods

Cell cultures

Spontaneously immortalized human thyroid cancer cells: 8505c human ATC cells harboring the hemi/homozygous BRAF^{V600E} mutation were purchased from DSMZ (German collection of microorganisms and cell culture, Braunschweig, Germany)[9; 27]. Human ATC SW1736 cells were provided by Dr. Nils-Erik Heldin (Uppsala University, Uppsala, Sweden), which harbor the heterozygous BRAF^{WT/V600E} mutation. Human thyroid cancer cells were grown in DMEM high glucose (CellGro, USA) medium supplemented with 10% fetal bovine serum (FBS) (CellGro, USA) and ampicillin/streptomycin. Primary human microvascular endothelial cells (blood vessel endothelial cells (BVECs) and lymphatic vessel endothelial cells (LVECs)) [28] were kindly provided from Dr. Harold F. Dvorak (BIDMC, Harvard Medical School, Boston, USA). BVECs and LVECs were grown in MCDB 131 (Life Technologies, USA) growth medium with additional glutgro (Corning, final concentration 2 mM) and MVGS (microvascular growth supplement) (Life Technologies, USA). For starvation conditions, MCDB 131 was supplemented with glutgro with 1% MVGS. All *in vitro* angiogenic and ELISA assays were performed on ATC cells cultured with the specific growth medium supplemented with no FBS.

Vemurafenib preparation

Vemurafenib (PLX4032, RG7204) (Roche/Genentech, NYC, USA) was dissolved in dimethyl sulfoxide (DMSO) (Sigma, USA) to achieve a stock concentration of 10 mM for *in vitro* assays. Intermediate doses of vemurafenib were diluted in 0.2% FBS DMEM high glucose in order to achieve final concentrations of 10 µM at 2% DMSO.

Western Blotting

Western blotting assays were performed according to a standard procedure, and the lysis buffer, composed of 10 mM Hepes (pH 7.40), 150 mM NaCl, 5 mM EDTA, 1 mM EGTA, 1 mM sodium vanadate, 5 mM sodium fluoride, and 1% Triton X-100; protease and phosphatase inhibitors (Pierce) were used for protein extractions [12]. The intensity of the bands was quantified with a densitometer (Quantity One 1-D analysis software, Bio-Rad, USA). The quantity of signal in the vemurafenib lane was divided by the signal of the vehicle lane in the corresponding tubulin or β -actin blot lane. We used the following antibodies: pERK1/2 (cat#9101, Cell Signaling, USA), total-ERK1/2, (cat#9102, Cell Signaling, USA), beta-actin or tubulin (Sigma, USA).

ELISA

ATC cells were cultured in 6-well dishes with DMEM high glucose growth medium with no FBS in the presence of 10 μ M vemurafenib dissolved in 2% DMSO or in 2% DMSO (control) for 24 hours. The next day, growth medium enriched by ATC-derived growth factors (secretome) was collected, separated from dead cell debris by short spin, diluted 1:3, and then tested in ELISA to measure secreted VEGFA, FGF β , EGF, Leptin, TNF α , IL6, IGF-1 and TGF β (Signosis, CA, USA), and VEGFC (Quantikine immunoassay, R&D Systems, MN, USA) according to the manufacturer's instructions. Protein concentration was measured using a standard curve. Protein levels were normalized to total protein content (μ g/ μ l) and growth medium which was used to determine subtracted background.

In vitro angiogenesis assay

In vitro angiogenesis assays were performed as previously described [29] [27]. In brief, BVECs or LVECs were starved overnight in growth medium with 1% serum. For *in vitro* angiogenesis tube formation assay, the endothelial cells (80×10^3) were suspended in ATC-derived secretome treated with vehicle (DMSO) or vemurafenib (10 μ M) with no FBS and seeded on growth factor-depleted Matrigel (cat#354230, BD Biosciences, USA). After about 5–6 hours of incubation, endothelial tubes were photographed. The number of tubes was counted using a 10 $\times$ or 20 $\times$ objective and four fields were chosen per well with two wells per each condition.

Histology and Immunohistochemistry

Histopathology evaluation of 6 ATC (3 ATC with *BRAF*^{V600E} and 3 ATC with *BRAF*^{WT}) was performed by a pathologist (P.S.) on hematoxylin and eosin (HE)-stained formalin-fixed paraffin-embedded (FFPE) tissues. For all patients we used discarded and unidentified tissues (IRB-approved, Massachusetts General Hospital). All tissue specimens were fixed with 10% buffered formalin phosphate and embedded in paraffin blocks. These were visualized with an Olympus BX41 microscope and the Olympus Q COLOR 5 photo camera (Olympus Corp., Lake Success, NY, USA). Sections (5 μ m thick) of ATC were classified according to the World Health Organization (WHO) [30] or ATC tissues (serial sections) were used for immunohistochemistry (IHC). After baking overnight at +37°C, deparaffinization with xylene/ethanol and rehydration were performed. IHC analysis was performed using primary antibodies (Supplementary Table 1). The sections, treated with

pressure cooker for antigen retrieval (Biocare Medical, Concord, CA, USA), were incubated at 123°C in citrate buffer (Dako Target Retrieval Solution, S1699; DAKO Corp., USA), cooled and washed with PBS. Antigen retrieval was performed for 60 min at room temperature. The primary antibody was detected using a biotin-free secondary antibody (K4011) (Dako Envision system, USA) and incubated for 30 min. All incubations were carried out in a humid chamber at room temperature. Slides were rinsed with PBS between incubations. Sections were developed using 3,3-diaminobenzidine (Sigma Chemical Co.) as a substrate and were counterstained with Mayer's hematoxylin [12] [31] [32]. The IHC markers immunoexpression was assessed semiquantitatively using the following scoring method: 0 (negative), 1+ (1–10% positive cells), 2+ (11–50% positive cells), and 3+ (more than 50% positive cells). Microvascular density is defined by number of vessels per microscope field showing CD31 (blood vessel endothelial cells) or D2-40 (Podoplanin) (lymphatic vessel endothelial cells) staining.

Statistical analysis

Statistical analysis was carried out using Excel software (version 2007, USA). *T-student* test was used to analyze the statistical significance of differences between two groups. For categorical data, Fisher's exact test was used. All reported *P* values were two-sided. Data are reported as the averaged value, and error bars represent the standard deviation or standard error mean of the average for each group in duplicate or triplicate. Results with *P* values below 0.05 were considered statistically significant.

Results

Protein expression of angiogenic/cachectic and pro-inflammatory factors in ATC cells with BRAF^{V600E}

We have quantitated microenvironment-associated angiogenic/cachectic and pro-inflammatory/immune response effectors of BRAF^{V600E} in the secretome derived from ATC cells by applying a multiplex ELISA assay (Figure 1). ATC cells were cultured *in vitro* in the presence of vemurafenib (10 µM) or vehicle DMSO for 24 hours. Ten µM vemurafenib was an effective dose to substantially block the BRAF^{V600E}-ERK1/2 pathway by specifically reducing phospho(p)ERK1/2 protein levels by about 90% or 50% in 8505c or SW1736 ATC cells, respectively, as compared to vehicle-treated (control) cells within 24 hours (Figure 2A). The secretome (growth medium enriched with ATC-derived angiogenic/cachectic factors) was collected and protein levels were determined by ELISA. We found that the homozygous BRAF^{V600E}-ATC 8505c cells secreted significantly (*p*<0.01, up to 182-fold) higher protein levels of angiogenic/cachectic factors VEGFA, TNFα, IGF1, and FGFβ compared to the heterozygous BRAF^{V600E} ATC SW1736 cells (Figure 1). In contrast, BRAF^{V600E} ATC SW1736 cells secreted significantly (*p*<0.001, up to 1.8-fold) higher protein levels of the lymphangiogenic factor VEGFC versus BRAF^{V600E}-ATC 8505c cells (Figure 1). Interestingly, only VEGFA (2-fold, *p*<0.01), VEGFC (7.5-fold, *p*<0.001) and IL6 (1.6-fold, *p*<0.01) protein levels were significantly down-regulated in the secretome derived from vemurafenib-treated ATC 8505c cells as compared to vehicle-treated ATC 8505c cells (Figure 2B). VEGFA, VEGFC and IL6 protein levels significantly decreased even stronger up to 18-fold (VEGFA, *p*<0.001), 3.2-fold (VEGFC, *p*<0.001), or 2.8-fold (IL6, *p*<0.01) in

the secretome from vemurafenib-treated ATC SW1736 cells as compared to vehicle-treated ATC SW1736 cells (Figure 2B).

Vemurafenib anti-BRAF^{V600E} therapy impairs *in vitro* angiogenesis in human microvascular endothelial cells

We found that BVECs formed tubules in a reconstituted microenvironment ECM basement membrane (Matrigel) when stimulated by either homozygous *BRAF*^{V600E} ATC 8505c (BVECs= 2-fold increase, *p*<0.01) (Figure 3A) or heterozygous SW1736 ATC cells (BVECs= 2-fold increase, *p*<0.01) (Figure 3B) derived secretome as compared with the growth medium (Figure 3C). Heterozygous *BRAF*^{WT/V600E} ATC SW1736 cells-derived secretome was even more potent in inducing LVECs tubule formation as compared to secretome derived from homozygous *BRAF*^{WT/V600E}-ATC 8505c cells (which were unable to induce lymphatic tubule formation, 23-fold in decrease, *p*<0.001) or growth medium (2-fold in decrease, *p*<0.01) (Figure 4A–C). Importantly, since vemurafenib is a selective inhibitor of BRAF^{V600E}, we found a significant decrease (*p*<0.001) in BVECs tubule formation of about 2.4-fold in the presence of secretome derived from vemurafenib-treated homozygous *BRAF*^{V600E} ATC 8505c cells or 3.5-fold in the presence of secretome derived from vemurafenib-treated *BRAF*^{WT/V600E} ATC SW1736 cells compared to vehicle-treated cells (Figure 3). LVECs tubule-formation was significantly inhibited up to 7-fold in the presence of vemurafenib-treated secretome derived from the heterozygous *BRAF*^{WT/V600E}-ATC SW1736 cells as compared to vehicle-treated SW1736 cells (Figure 4A–C).

***BRAF*^{V600E}-ATC are enriched in microvascular endothelial cells and up-regulate secretion of angiogenic and cachectic molecules**

Tumor angiogenesis is functionally related to endothelial cells; indeed, we assessed microenvironment-associated vascular and lymphatic endothelial cells which participate in the aberrant behavior of ATC with *BRAF*^{V600E} or *BRAF*^{WT} (Figure 5). We found that VEGFA, VEGFC and IL6 protein levels were significantly higher (10-fold increase, *p*<0.01) in *BRAF*^{V600E}-ATC as compared to *BRAF*^{WT}-ATC (Figure 5). Moreover, *BRAF*^{V600E}-ATC showed significantly higher intratumoral microvascular density identified by CD31 immunohistochemical stain (Figure 5, *p*<0.01).

Discussion

Experimental models of ATC are based on dedifferentiation from well-differentiated thyroid cancer subtypes [10; 14; 33] [34]. Diverse pathways are altered in ATC, including at least the RAS/RAF/MAPK and PI3K/AKT signaling cascades [8] [9] [7] [35] [36] [14] [37] [38; 39; 40]. We previously found that BRAF^{V600E} regulates protein expression of extracellular matrix (ECM) molecules in human ATC cells which are required for tumor invasion and metastasis [12; 41; 42]. Tumor-associated angiogenesis is a pathologically complex process which involves recruitment of new endothelial cells and their assembly into tubes (vasculogenesis) in addition to the sprouting of new vessels from existing ‘mother’ vessels [43]. Increased tumor-associated angiogenesis is crucial for maintaining tumor cell growth, migration, invasion and metastasis [44]. Overproduction of angiogenic factors is referred to as the angiogenic switch, and those molecules are also involved in cachexia evolution and

pro-inflammatory mechanisms. Therefore, the angiogenic switch and emerging cachexia could represent an important crossroad in the evolution of human cancer [45]. Advanced cancers, such as ATC, show a pleomorphic and interconnected network between high-grade malignant cells and stromal cells (e.g., macrophages) that characterize a tumor microenvironment which might contribute to pathologic angiogenesis by the production of angiogenic/cachectic factors and pro-inflammatory/immune response cytokines (e.g. interleukins, etc.) [46; 47]. Also, the orthotopic human BRAF^{V600E}-positive 8505c ATC microenvironment model *in vivo* contains macrophages that may contribute to the ATC aggressiveness [41]. Undifferentiated human cancer cells secrete IL6, Leukemia inhibitor factor (LIF), TNF α , and TGF β *in vitro*, which have all been connected to tumor growth in ATC [48]. PTC more frequently metastasize to neck lymph nodes and less frequently to distant sites excluding the oncocytic variant [49]; whereas, ATC show regional and distant metastases [50]. The most common site of metastasis in patients with ATC is lung (78%), followed by intrathoracic lymph nodes (58%), neck lymph nodes (51%), pleura (29%), adrenal glands (24%), liver (20%), brain (18%), and heart (18%) [50]. Some angiogenic markers have been studied in PTC [27; 51], here we have found that undifferentiated/anaplastic cells secreted different angiogenic/cachectic and pro-inflammatory factors (e.g. VEGFA, VEGFC and IL6) which triggered *in vitro* angiogenesis (tubule formation). Intriguingly, ATC cells with heterozygous BRAF^{V600E} tend to induce lymphatic endothelial cells tubule formation *in vitro*, perhaps because of the higher secreted VEGFC protein levels as compared to homozygous BRAF^{V600E} ATC cells. This result could suggest an oncogenic dosage-dependency in the regulation of angiogenesis. Also, the tumor-associated endothelial cell population could be biologically heterogeneous and elicit different dynamic processes which affect their paracrine communications with ATC cells in the microenvironment. This result will require further studies to elucidate the specific role of BRAF^{V600E} in the regulation of angiogenesis versus lymphangiogenesis. Also, human clinical samples of ATC with BRAF^{V600E} compared to BRAF^{WT}-ATC tend to recruit blood microvascular endothelial cells in the intratumoral area, suggesting that increased ATC-associated angiogenesis might play a role to supply energy/nutrients and support intravascular invasion and ultimately metastasis. It is widely known that VEGFA and VEGFC are potent angiogenic and lymphangiogenic factors [52], respectively, which regulate blood and lymphatic vessels endothelial cell migration and proliferation through the VEGFR2 (vascular endothelial growth factor receptor 2) intracellular signaling cascade [28]. VEGFA [53] and VEGFC [51] have been reported to be highly expressed in PTC over normal thyroid tissue or benign lesions and correlate with increased angiogenesis and lymphatic vessel density [51; 53; 54; 55]. Moreover, IL6 is up-regulated by BRAF^{V600E} mutation in melanoma [56] and by RET/PTC translocations in thyroid cancer [57] and increased in chronic inflammatory disease and many cancers, including PTC [58; 59]. IL6 plays a fundamental role as a pro-inflammatory molecule through ERK1/2, JAK/STAT3 or PI3K/AKT intracellular signaling cascades [60].

More importantly, our results show that 10 μ M vemurafenib anti-BRAF^{V600E} therapy effectively down-regulated protein levels of IL6, VEGFA, and VEGFC and inhibited *in vitro* angiogenesis and lymphangiogenesis. These findings suggest that BRAF^{V600E} could regulate microenvironment communication between ATC and endothelial cells which potentiate

tumor migration, invasion, and metastasis, facilitating tumor progression. Doses greater than 1 μM (e.g. 2 or 10 μM) of vemurafenib have also been reported to be effective in *BRAF*^{V600E}-melanoma or ATC-derived cells *in vitro* and block intracellular signaling pathways required for tumor proliferation [61] [62]. Also, our previous studies have set 10 μM PLX4720 as an effective dose to inhibit thyroid cancer cell migration and invasion [42]. Furthermore, megestrol acetate, approved by the FDA (Food and Drug Administration) in 1993, is used to improve appetite and increase weight gain in patients with cancer-related cachexia; however, side effects such as edema, thromboembolic phenomena and poor survival have limited its utility [63] [34]. Moreover, clodronate reduced ATC cell proliferation and cytokine production in a time/dose-dependent manner. A decrease in serum IL6, TNF α and granulocyte colony stimulating factor were shown. Liver necrosis was also decreased during administration of the drug; however, the discontinuation treatment resulted in tumor regrowth and weight loss [64]. Collectively, those data suggest that new therapeutic options against cancer cachexia are urgently needed. In *in vivo* orthotopic mouse models of *BRAF*^{V600E}-positive human ATC 8505c cells, PLX4720 (a selective orally available inhibitor of *BRAF*^{V600E}) has been shown to not only block progression of cachexia but also reverse the process, inhibit lung metastases and prolong animal survival [26], suggesting that anti-*BRAF*^{V600E} therapy might be in some way helpful to block production of some pro-cachectic factors at least in patients with *BRAF*^{V600E}-positive carcinomas. Furthermore, it has been shown that pharmacological inhibition of FOXM1 (member of the forkhead box family of transcription factors involved in control of cell proliferation, chromosomal stability, angiogenesis, and invasion) with thiostrepton treatment reduced *BRAF*^{V600E}-positive ATC 8505c cell growth and metastasis *in vivo* [18] and might delay cachexia. Further studies will be needed to better understand molecular mechanisms of ATC-related cachexia and crosstalk with tumor metabolism and the angiogenic microenvironment. Also, for potential applications of anti-*BRAF*^{V600E} targeted therapies directed toward cachexia prevention, side effects of *BRAF*^{V600E} inhibitors [65] must be considered in order to determine their potential in patients with advanced thyroid cancer and a multi-institutional cohort will be needed to capture a sufficient number of patients with this type of cancer.

In summary, our results suggest that VEGFA, VEGFC and IL6 are angiogenic/cachectic or pro-inflammatory/immune response factors which could play a crucial role in *BRAF*^{V600E}-positive human ATC (Figure 6). Understanding the extent to which microenvironment-associated angiogenic factors participate in cachexia and cancer metabolism in advanced thyroid malignancies will reveal new biomarkers and foster potential alternative therapeutic approaches. Anti-angiogenic therapy may be an additional rational adjuvant therapeutic modality for use in patients with advanced thyroid cancers and cachexia-associated clinical manifestations.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

Cachexia is the result of complex metabolic alterations which causes morbidity in patients with advanced cancers including undifferentiated (anaplastic) thyroid carcinoma (ATC). ATC is a lethal disease with limited therapeutic options and unclear etiology for cachexia. Our results suggest that angiogenic/cachectic and pro-inflammatory/immune response factors could play a crucial role in *BRAF*^{V600E}-positive human ATC aggressiveness. Understanding the extent to which microenvironment-associated angiogenic factors participate in cachexia and cancer metabolism in advanced thyroid cancers will reveal new biomarkers and foster novel therapeutic approaches.

Figure 1. Measurements of angiogenic/cachectic and pro-inflammatory factors secreted by ATC cells with homozygous (i.e. 8505c) or heterozygous (i.e. SW1736) *BRAF*^{V600E} mutation

Measurements of VEGFA, VEGFC, TNF α , IGF1, FGF β , EGF, TGF β , Leptin and IL6 secreted protein levels in the vehicle-treated secretome derived from homozygous *BRAF*^{V600E} ATC (anaplastic thyroid carcinoma) 8505c or heterozygous SW1736 ATC cells cultured *in vitro* with no serum (FBS) for 24 hours. The secretome (growth medium enriched with ATC cells-derived angiogenic/cachectic factors) was collected and protein levels (pg/mL) were determined by ELISA (enzyme-linked immunosorbent assay). Protein levels were normalized to total protein content ($\mu\text{g}/\mu\text{L}$) and to growth medium which was used to determine subtracted background. These data represent the average $\pm$ standard deviation (error bars) of 2 independent replicate measurements (* $p<0.05$, ** $p<0.01$, *** $p<0.001$).

Figure 2. Measurements of VEGFA, VEGFC, and IL6 angiogenic/cachectic factors secreted by ATC cells with homozygous (i.e. 8505c) or heterozygous (i.e. SW1736) *BRAF*^{V600E} mutation

A) A parallel plate similar to B was set up and corresponding phospho(p)ERK1/2 protein levels (low exp= shorter exposure during chemiluminescence reaction; high exp= longer exposure during chemiluminescence reaction) measured by western blotting from *BRAF*^{V600E}-ATC (anaplastic thyroid carcinoma) 8505c or SW1736 adherent cells grown for 24 hrs in the presence of DMSO (dimethyl sulfoxide) (vehicle; control) or 10 μ M vemurafenib (vemu). B) Measurements of VEGFA, VEGFC, and IL6 secreted protein levels in the vehicle-treated secretome derived from homozygous *BRAF*^{V600E} ATC 8505c or heterozygous *BRAF*^{V600E} SW1736 ATC cells cultured *in vitro* with no serum (FBS) in the presence of DMSO (vehicle; control) or 10 μ M vemurafenib for 24 hours. The secretome (growth medium enriched with ATC cells-derived angiogenic/cachectic factors) was collected and protein levels (pg/mL) were determined by ELISA (enzyme-linked immunosorbent assay). Protein levels were normalized to total protein content (μ g/ μ L) and to growth medium which was used to determine subtracted background. These data represent the average $\pm$ standard deviation (error bars) of two independent replicate measurements (* p <0.05, ** p <0.01).

Figure 3. Vemurafenib inhibits blood vessel endothelial cells-related *in vitro* angiogenesis triggered by $BRAF^{V600E}$ -ATC cell derived secretome

A–B) Secretome from homozygous or heterozygous $BRAF^{V600E}$ -positive ATC (anaplastic thyroid carcinoma) cells triggered human blood microvascular endothelial cells (BVECs) tube formation *in vitro*. BVECs (with starvation overnight at 1% serum) were suspended in secretome (CM: conditioning medium) derived from 8505c or SW1736 cells with $BRAF^{V600E}$ treated with vehicle or vemurafenib (10 μ M) for 24 hours in the absence of serum and seeded on growth factor–reduced Matrigel. The secretome was utilized to induce tube formation. BVECs tubes were photographed after 5–6 hours. Magnification: 10 $\times$. C) *In vitro* quantitation of microvascular endothelial cell tube formation: histograms show significant changes in BVECs tubules formation triggered by 8505c or SW1736 ATC^{BRAFV600E}-derived vehicle-treated secretome as compared to vemurafenib-treated secretome. These data represent the average $\pm$ standard deviation (error bars) of replicates from two independent experiments (* p <0.05, ** p <0.01, *** p <0.001).

Figure 4. Vemurafenib inhibits lymphatic vessel endothelial cells-related *in vitro* angiogenesis triggered by heterozygous *BRAF*^{V600E}-ATC cell derived secretome
A–B) Secretome from homozygous or heterozygous *BRAF*^{V600E}-positive ATC (anaplastic thyroid carcinoma) cells triggered human lymphatic microvascular endothelial cells (LVECs) tube formation *in vitro*. LVECs (with starvation overnight at 1% serum) were suspended in secretome (CM: conditioning medium) derived from 8505c or SW1736 cells with *BRAF*^{V600E} treated with vehicle or vemurafenib (10 μ M) for 24 hours in the absence of serum and seeded on growth factor–reduced Matrigel. The secretome was utilized to induce tube formation. LVECs tubes were photographed after 5–6 hours. Magnification: 10 $\times$. C) *In vitro* quantitation of microvascular endothelial cell tube formation: histograms show significant changes in LVECs tubules formation triggered by SW1736 ATC^{*BRAF*^{V600E}}-derived vemurafenib-treated secretome as compared to vehicle-treated secretome. These data represent the average $\pm$ standard deviation (error bars) of replicates from two independent experiments (* p <0.05, ** p <0.01, *** p <0.001).

Figure 5. Immunohistochemical staining of angiogenic/cachectic and pro-inflammatory factors in ATC samples with or without the *BRAF*^{V600E} mutation

A) Hematoxylin and eosin (HE) staining of ATC. HE images correspond with foci shown with CD31, D2-40, VEGFA, VEGFC and IL6 antibodies. Tumor cells are epithelioid to spindled with hyperchromatic nuclei and eosinophilic cytoplasm. The CD31 antibody significantly stains (arrows) intratumoral endothelial cells in ATC (anaplastic thyroid carcinoma) with *BRAF*^{V600E} (n=3) as compared to ATC with *BRAF*^{WT} (n=3). Podoplanin protein expression is not detected in the ATC either with *BRAF*^{V600E} or *BRAF*^{WT}. The VEGFA antibody shows strong cytoplasmic staining of tumor cells in ATC with *BRAF*^{V600E} (n=3) (scoring: 3+) compared to ATC with *BRAF*^{WT} (n=3) (scoring: 2+; cytoplasmic). VEGFC antibody diffusely stains cytoplasm (scoring: 2+) and nuclei (weak; 1+) of tumor cells in ATC with *BRAF*^{V600E} (n=3) (asterisk marks the ATC and arrows mark papillary thyroid carcinoma which are positive) compared to ATC with *BRAF*^{WT} (n=3) (scoring: 1+, cytoplasm). IL6 antibody shows strong staining (scoring: 3+) of stromal cells and moderate staining (scoring: 2+) of tumor cells in ATC with *BRAF*^{V600E} (n=3) compared to ATC with *BRAF*^{WT} (n=3) which show weak cytoplasmic/membranous staining (scoring: 1+).

B) Microvascular density is defined by number of vessels per microscope field exhibiting CD31 or D2-40 (Podoplanin) staining. *BRAF*^{V600E}-ATC show greater intratumoral staining for CD31 as compared with *BRAF*^{WT}-ATC. These data represent the average $\pm$ standard deviation (error bars) of 6 ATC samples (*p<0.05, **p<0.01, ***p<0.001).

Figure 6. Expression of potential angiogenic/cachectic and pro-inflammatory factors expressed in human anaplastic thyroid cancer

Secretion of different angiogenic/cachectic and pro-inflammatory factors (e.g. VEGFA, TNF α , IGF1, FGF β , EGF, TGF β , Leptin, IL6, etc.) by anaplastic thyroid carcinoma (ATC).