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Tyrosine kinase inhibitor therapy and metabolic remodelling in papillary thyroid cancer

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Abstract

Targeted therapy is increasingly used to manage metastatic papillary thyroid cancer. The focus of the present study was to examine glucose metabolism and tumor responses for thyroid cancer xenografts expressing the glycolytic pathway modulators platelet-derived growth factor receptor (PDGFR) and BRAF^{V600E}. Radiolabelled glucose derivative [¹⁸F]FDG was used to analyze the effects of PDGFR blockade with imatinib, BRAF blockade with vemurafenib, as well as combined PDGFR and BRAF blockade *in vitro* and *in vivo* with PET. Dynamic PET data was correlated with immunohistochemistry staining and kinetic analysis for facilitative glucose transporter 1 (GLUT1) and hexokinase-II (HK2). Vemurafenib decreased [¹⁸F]FDG uptake in BCPAP cells *in vitro*; however, it was increased by ~70% with imatinib application to BCPAP cells. This metabolic response to tyrosine kinase inhibition required BRAF^{V600E} as it was not seen in cell lines lacking mutated BRAF (TPC1). In xenografts, imatinib therapy in BCPAP thyroid tumour-bearing mice significantly increased [¹⁸F]FDG uptake and retention (>30%) in BCPAP tumours with PDGFR β or both ($\alpha+\beta$) isoforms. Kinetic analysis revealed that the increased glucose uptake is a consequence of increased phosphorylation and intracellular trapping of [¹⁸F]FDG confirmed by an increase in HK2 protein expression and activity, but not GLUT1 activity. BRAF inhibition alone, or combined PDGFR and BRAF inhibition, reduced (~60%) [¹⁸F]FDG uptake in both types of BCPAP (β or $\alpha+\beta$) tumours. In terms of tumour growth, combination therapy with imatinib and vemurafenib led to a near abolition of the tumors (~90% reduction), but single therapy for BCPAP with PDGFR α expression was much less effective. In summary, imatinib led to a paradoxical increase of [¹⁸F]FDG uptake in xenografts that was reversed through BRAF^{V600E} inhibition. The present data show that metabolic reprogramming in thyroid cancer occurs as a consequence of BRAF-mediated upregulation of HK2 expression that may permit tumour growth with isolated blockade of upstream tyrosine kinase receptors.

Key Words

- thyroid cancer
- [¹⁸F]FDG PET
- Imatinib
- Vemurafenib
- BRAF mutation
- PDGFR

Endocrine-Related Cancer
(2020) 27, 495–507

Introduction

Papillary thyroid cancer (PTC) is the most common type of thyroid cancer. PTC, in the majority of cases, will respond to a combination of surgery and radioactive iodine for treatment of metastatic disease. However, approximately 15% of thyroid cancer patients are predisposed to a poor prognosis due to locally advanced or diffuse metastatic disease (Matrone *et al.* 2017). For these patients, there has been a significant attempt to identify suitable targeted therapies. More than 15 studies have examined different tyrosine kinase inhibitors in an effort to determine effective drug regimens for patients with iodine-refractory disease (Durante *et al.* 2006, Matrone *et al.* 2017, Valerio *et al.* 2017). Since most of these drugs target multiple receptor classes including vascular endothelial growth factor receptor (VEGFR), c-KIT, re-arranged during transfection receptor (RET), and platelet-derived growth factor receptor (PDGFR α/β), the clinical trial results demonstrate mixed efficacy and are difficult to compare between the different tyrosine kinase inhibitors. Targeted inhibition of tyrosine kinase receptors such as PDGFR or VEGFR can induce compensatory responses in pERK and other pathways that may abrogate the effects of TKI treatment (Ekpe-Adewuyi *et al.* 2016, Lopez-Campistrous *et al.* 2016). The latter study found that pharmacological and antibody-mediated blockade of PDGFR α led to an upregulation of pERK expression in BCPAP cells. Moreover, many of those drugs have exhibited considerable side-effect profiles that lead to substantial patient drop-out rates and limited use for patients with metastatic, but slowly progressing, disease (Klein Hesselink *et al.* 2015, Viola *et al.* 2016, Liu *et al.* 2018).

Patient selection and the application of tyrosine kinase inhibitors for thyroid cancer, such as imatinib, lenvatinib, or sorafenib, remain empiric in nature. However, preclinical and clinical research is still ongoing using other TKIs to determine their specific efficacy and how the multi-targeting compounds result in a specific overall effect on thyroid cancer cells *in vivo*. The ability to assess TKI therapy efficacy remains limited. Non-invasive molecular imaging techniques such as PET using [^{18}F]FDG have been often applied to analyse effects of tyrosine kinase inhibitors *in vivo*. For solid tumours, [^{18}F]FDG-PET represents the 'gold standard' radiotracer for oncologic imaging and it is used extensively for monitoring response to therapy (Farwell *et al.* 2014, Sheikhbahaei *et al.* 2017). Tyrosine kinase inhibitors are known to suppress growth-factor-driven glycolysis in cancer (Poliaková *et al.* 2018), implying that the glucose derivative [^{18}F]FDG-PET can be

used to quantify its biological response *in vivo*. The success of receptor kinase blockade has been monitored at an early stage with [^{18}F]FDG-PET imaging in many forms of cancer including lung (Ma *et al.* 2017), melanoma (Wong *et al.* 2017), gastrointestinal stromal tumours (Van den Abbeele *et al.* 2012) and renal cell carcinoma (Vercellino *et al.* 2009, Ueno *et al.* 2012). While many studies have examined the role of PET imaging for diagnosing thyroid cancer, few studies have examined its use in monitoring therapy outside of small numbers in clinical trials (Marcus *et al.* 2014, Haugen *et al.* 2016, Takeuchi *et al.* 2018). It is known that increased [^{18}F]FDG uptake into primary thyroid tumours and metastases correlates with dedifferentiation, the presence of the BRAF mutation, and poor prognosis (Nagarajah *et al.* 2015, Yang *et al.* 2017, Santhanam *et al.* 2018). However, [^{18}F]FDG uptake into thyroid tumour lesions is highly variable and does not always correlate with severity of disease. Benign neoplasms may also often demonstrate an impressive glycolytic metabolic activity (Barrio *et al.* 2016). Thus, [^{18}F]FDG-PET scans in thyroid cancer have been used selectively given the lack of understanding of glucose metabolism in this patient population.

Platelet-derived growth factor receptor is a tyrosine kinase receptor with two isoforms alpha (α) and beta (β). Upon binding of PDGF ligands (AA-, BB- CC-, DD-), the receptors dimerize ($\alpha\alpha$, $\alpha\beta$, $\beta\beta$) to activate downstream signalling pathways. PDGFR β can be expressed in benign and malignant thyroid neoplasm but the PDGFR α , and its associated cytokine ligand PDGF-AA, is linked to metastatic disease in thyroid cancer and may act as control regulator for cell dedifferentiation and resistance to radioactive iodine (Ekpe-Adewuyi *et al.* 2016, Lopez-Campistrous *et al.* 2016). PDGFR α activates the Akt, ERK, and STAT3 pathways in thyroid cancer and may also control glycolytic activity as demonstrated for other types of cancer including gastrointestinal stromal tumors (GIST) (Ran *et al.* 2013). The focus of the present study was to explore the potential functional link between oncogenic kinase drivers PDGFR α/β and the proto-oncogene BRAF^{V600E}, and glucose metabolism in thyroid cancer. We also examined the influence of tyrosine kinase inhibitor therapy in a thyroid xenograft model with BCPAP (BRAF^{V600E} mutation) that has native (PDGFR β only) or engineered (PDGFR $\beta+\alpha$) proteins expressed. In addition, the human papillary thyroid carcinoma cell line (TPC1) was used as a positive control expressing both PDGFR $\beta+\alpha$ and WT BRAF lacking V600E mutation. The goal was to analyse the role of PDGFR and BRAF signalling in glucose uptake and metabolism when cells

are subjected to growth factor receptor inhibition, with and without blockade of BRAF^{V600E} and in comparison to BRAF blockade alone. [¹⁸F]FDG-PET was used as a non-invasive tool to examine tumour glucose metabolism and the functional analysis of effects of PDGFR blockade alone with imatinib, with vemurafenib or in combination with BRAF inhibitor, vemurafenib.

Materials and methods

General

All chemicals and reagents were obtained from MilliporeSigma unless otherwise stated. Imatinib mesylate (amorphous form) and vemurafenib were obtained from Astatech Inc. (Bristol, PA, USA). Supersaturated solutions of imatinib mesylate in saline were freshly prepared for each animal experiments. Human recombinant PDGF-AA was obtained from Gibco (ThermoFisher Scientific). Doxycycline was purchased from Sigma-Aldrich Inc. Doxycycline (0.5 mg/21 days) slow-releasing pellets for animal experiments were from Innovative Research of America (Sarasota, FL, USA). Radiotracer [¹⁸F] FDG was prepared at the Edmonton PET Center of the Cross Cancer Institute using a GE TracerLab MX automated synthesis unit (GE Healthcare Canada Inc.) according to established synthesis procedures (Hamacher *et al.* 1986).

Cell culture

PDGFR β native and PDGFR α + β BCPAP cell lines were generated as previously described (Lopez-Campistrous *et al.* 2016). Briefly, BCPAP cells which had been obtained from the German Collection of Microorganisms and Cell Cultures GmbH (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, DSMZ; Braunschweig, Germany) were subjected to lentiviral transduction with doxycycline-inducible retrovirus system (Lenti-X Lentiviral Expression Systems; Clontech Laboratories Inc.) to express PDGFR α or mock protein. For WT BRAF control, TPC1 cells were used (Lopez-Campistrous *et al.* 2016). The cells were cultured as previously described (Wagner *et al.* 2018) using high glucose medium (DMEM with HEPES and L-glutamine) supplemented with 5 vol% foetal bovine serum (FBS) in presence of 100 units/mL of penicillin and 100 μ g/mL of streptomycin. To induce protein synthesis, doxycycline (2 μ g/mL) was added at least 48 h before each *in vitro* experiment.

Cellular uptake studies

PDGFR β or α + β BCPAP (75,000 cells) with BRAF^{V600E} mutation, or TPC1 (60,000 cells) as BRAF WT control, were seeded 48 h before the experiment with medium containing PDGF-AA (50 ng/mL) and doxycycline (2 μ g/mL). 0.3 μ M imatinib was added if indicated. [¹⁸F]FDG uptake experiments were conducted in glucose free Krebs buffer with 1 h preincubation. Cells were incubated for the indicated time points, washed three times with PBS and lysed with RIPA buffer. Radioactivity was measured on a PerkinElmer Wizard² 2480 Automatic Gamma Counter (Perkin Elmer). The radioactivity was normalized to the protein content in the cell lysate by using the Pierce BCA Protein Assay Kit (Pierce/ThermoFisher Scientific). Each experiment was done in triplicate using three wells each.

Animals

All animal experiments were conducted under the necessary ethical approval of the animal care committee of the Cross Cancer Institute. Male NSG mice were obtained from a breeding colony from Dr Lynne Postovit (Department of Oncology, University of Alberta, Edmonton, Canada). Around 3-month-old NSG mice were injected with 2.5×10^6 PDGFR β expressing BCPAP cells in 100 μ L PBS/Matrigel into the left shoulder. One week later, 2.5×10^6 PDGFR α + β expressing cells also in 100 μ L PBS/Matrigel were injected into the right shoulder. This staggered xenograft BCPAP tumour growth timing was required because of the much more rapid growth of BCPAP PDGFR α (+) tumours compared to the xenografts with only PDGFR β . A doxycycline slow-releasing pellet was implanted into the right flank at the same time. One week later, mice were assigned into separate cages housing control and treatment groups. Tumors were palpable at that time. Mice were treated daily intraperitoneally with 50 mg/kg imatinib mesylate in 100 μ L saline, while the control animals received saline i.p. injections only for 6 days. Treatment was repeated with a different set of animals to verify the results and increase the total numbers. For vemurafenib therapy alone, mice were treated daily intraperitoneally with 20 mg/kg vemurafenib in 85 μ L DMSO/saline (80/20 v/v). The combination therapy of vemurafenib and imatinib was commenced as follows: Mice were treated daily intraperitoneally with 30 mg/kg imatinib mesylate in 85 μ L saline and 20 mg/kg vemurafenib in 85 μ L DMSO/saline (80/20 v/v), while the control animal received 85 μ L DMSO/saline (80/20 v/v) for 6 days. The mice underwent PET imaging with [¹⁸F]FDG on the last day of treatment

(control day 5, treatment group day 6). Tumour sizes were measured using a calliper before and on the last day of therapy. Tumor volumes were calculated from two dimensions and calculated using the formula for the volume: $V = (W^2 \times L)/2$ (Faustino *et al.* 2013).

In vivo PET imaging

PET imaging experiments were carried out on an INVEON® PET scanner (Siemens Preclinical Solutions, Knoxville, TN, USA). Mice were anesthetized by inhalation of isoflurane in 40% O₂/60% N₂, 1.5 L/min, while maintaining body temperature at 37 °C throughout the experiment. Five to 10 MBq of [¹⁸F]FDG in saline was administered intravenously using a tail vein catheter. Dynamic PET scans were acquired over 1 h post injection (p.i.). The image files were reconstructed using the maximum a posteriori (MAP) algorithm. The image files were further analysed using the ROVER v 2.0.51 software (ABX GmbH, Radeberg, Germany). Masks for defining 3D regions of interest (ROI) were delineated, and radioactivity in the ROIs was analysed by 50% thresholding. Mean standardized uptake values ($SUV_{mean} = (\text{activity/mL tissue})/(\text{injected activity/body weight})$, mL/g) were calculated for each ROI.

Western blot analysis of xenograft tumour samples

Excised xenograft tumours were processed using a gentleMACS™ Dissociator (Miltenyi Biotec, San Diego, CA, USA) in RIPA buffer (150 mM NaCl, 100 mM Tris (pH 8.0), 1% Triton X-100, 1% deoxycholic acid, 0.1% SDS, 5 mM EDTA, and 10 mM NaF) supplemented with 1 mM sodium vanadate, 2 mM leupeptin, 2 mM aprotinin, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM DTT, 2 mM pepstatin, and 1:100 protease inhibitor cocktail set III on ice. After centrifugation at 4°C at 35,000 *g* for 15 min, the supernatant was harvested as the total soluble protein extract. Protein concentration was determined using Pierce™ BCA Protein Assay Kit (Pierce Biotechnology). Protein extract samples (100 µg) were resolved by SDS-PAGE and transferred to nitrocellulose membrane, blocked in 5% non-fat milk in TBS containing 0.05% Tween 20 for 60 min, followed by incubation with primary antibodies at 4°C overnight. Protein bands were detected by incubation with horseradish peroxidase-conjugated antibodies (Pierce Biotechnology) and visualized with SuperSignal West Pico chemiluminescence substrate (Thermo Scientific). The following primary antibodies were used: PDGFRα (Cell signaling 5241; dilution 1:1000), p-ERK (Cell Signaling 9106; dilution 1:1000), t-ERK

(Santa Cruz (sc-94; dilution 1:2000) and γ-tubulin (Sigma-Aldrich T6557; dilution 1:10,000).

Immunohistochemistry

Excised xenograft tumour tissues were fixed in neutral buffered 10% formalin overnight and embedded into paraffin. Slides with 4µm sections were dried at 60°C for 1 h and re-hydrated by three changes of xylene for 10 min each, then graded ethanol from 100% to 50%, followed by water and TBS. Slides were microwaved in a pressure cooker for 6 min in citraconic anhydride (0.05% in water, pH 7.4) for antigen retrieval and blocked with 0.5% fish gelatin in Tris-buffered saline with 0.05% Tween 20 (TBST) for 30 min and incubated with mouse monoclonal anti-GLUT1 IgG1 (clone A-4, sc-377228, Santa Cruz Biotechnology, 1:50) or mouse monoclonal anti-HK-2 IgG1 (ab-37593, Abcam PLC Biotechnology, 1:50) in a humidity chamber overnight at 4°C. After incubation in 3% H₂O₂ in water for 15 min, slides were incubated with DakoCytomation Envision+ anti-mouse or anti-rabbit labelled Polymer HRP (DakoCytomation) for 1 h, developed, using Dako Liquid DAB+ Substrate Chromagen System and 1% copper sulphate and counterstained with hematoxylin. Slides were dehydrated by reversing rehydration and cover-slipped. Negative controls were prepared without primary antibodies. Quantification analysis of protein expression was carried out with ImageJ 1.x (NIH and LOCI, University of Wisconsin, Madison, WI, USA) and IHC profiler (Varghese *et al.* 2014).

Kinetic modelling

The dynamic PET data sets were analysed as previously described using a reversible two-compartment catenary model with blood volume parameter (Van den Hoff 2005, Prenen *et al.* 2006, Jans *et al.* 2018). For this purpose, an ROI defining the vena cava was generated by thresholding averaged early time frames (8–20 s p.i.) that visualized the initial bolus blood flow. The plasma input time-activity curves (TAC) generated from this ROI were convolved with the impulse response of the compartmental model and numerically fitted to the tumor-derived TAC of the same animal. This procedure was implemented in Matlab software (version R2018a), utilizing a Nelder–Mead simplex direct search. The fit was governed by the minimization of a cost function sensitive to the difference between measured values and those predicted by the model and the goodness of fit was determined by its *R*² value.

Statistical analysis

All *in vitro* data, semi-quantified PET as well as quantified protein expression data are expressed as mean $\pm$ S.E.M. Graphs and time-activity curves (TAC) were constructed using GraphPad Prism 5.0 (GraphPad Software). Statistical differences were tested by Student's *t*-test and were considered significant for $P < 0.05$ (*), $P < 0.01$ (**) and $P < 0.001$ (***).

Results

In vitro studies

The BRAF^{V600E} mutated thyroid cancer BCPAP cell line, both mock and PDGFR α transfected, showed a strong [¹⁸F]FDG *in vitro* cell uptake reaching 522 ± 23 in PDGFR β and $569 \pm 25\%$ radioactivity/mg protein in PDGFR $\alpha + \beta$ BCPAP cells after 120 min (Fig. 1A). This is consistent with the known strong dependence on glycolysis of the parental cell line (Coelho *et al.* 2016). Protein expression of PDGFR α has significant effects on cell morphology,

the epithelial-mesenchymal transition and xenograft tumour growth, but minimal influence on basic cellular [¹⁸F]FDG uptake (22–24). Surprisingly, a low dose treatment with 0.3 μ M imatinib over 48 h increased the [¹⁸F]FDG uptake *in vitro* by nearly 70% to $874 \pm 36\%$ radioactivity/mg protein ($P < 0.01$) in PDGFR β cells at 120 min while having minimal effect on cell count. In contrast, in a control experiment using the TPC1 thyroid cancer cell line lacking BRAF^{V600E}, imatinib treatment had no significant effect on the [¹⁸F]FDG uptake with 1064 ± 41 (no imatinib) and $1117 \pm 41\%$ radioactivity/mg protein (with 0.3 μ M imatinib) at 120 min (Fig. 1B). In contrast to the effects of imatinib treatment of PDGFR $\alpha + \beta$ and PDGFR β BCPAP cells, treatment with 0.25 μ M vemurafenib, an ATP competitive inhibitor targeting BRAF^{V600E}, strongly decreased *in vitro* cell uptake of [¹⁸F]FDG; 10 ± 1 in PDGFR β and $81 \pm 38\%$ radioactivity/mg protein ($P < 0.001$) in PDGFR $\alpha + \beta$ BCPAP cells after 120 min (Fig. 1A). Taken together, blocking PDGFR with imatinib leads to a strong increase in glucose metabolic demand, while blocking BRAF^{V600E} with vemurafenib strongly reduces glucose energy metabolism in BCPAP cells. PDGFR α expression in these cells had no influence on glucose demand. Similar to the results with [¹⁸F]FDG, there was also no influence of PDGFR α expression on [¹⁸F]FLT cell uptake (data not shown) indicating that proliferation rate was not influenced by PDGFR α .

In vivo PET imaging

Mouse xenografts bearing bilateral PDGFR β or $\alpha + \beta$ transfected BCPAP tumours were analysed with [¹⁸F]FDG PET. Figure 2 shows representative PET images at 60 min p.i. of [¹⁸F]FDG (Fig. 2A), the dynamic uptake profile over 60 min in tumour tissue and non-targeting muscle tissue (Fig. 2B) and the SUV_{mean} values after 60 min p.i. for visualization of the quantitative differences for each measured condition (Fig. 2C). Both, PDGFR β and PDGFR $\alpha + \beta$ tumours showed similar uptake levels and profiles over time reaching similar SUV values after 60 min p.i.: 1.88 ± 0.07 in PDGFR β and 1.85 ± 0.14 in PDGFR $\alpha + \beta$ tumours ($n = 13$). After 6 days of imatinib therapy (50 mg/kg daily), [¹⁸F]FDG uptake was significantly increased in both tumours with a higher uptake in PDGFR $\alpha + \beta$ tumours: SUV_{mean,60 min} 2.42 ± 0.11 ($n = 9$; $P < 0.001$) in PDGFR β and 2.69 ± 0.28 ($n = 9$; $P < 0.01$) in PDGFR $\alpha + \beta$. In contrast, 6 days of a single vemurafenib therapy (20 mg/kg daily) strongly reduced [¹⁸F]FDG tumour uptake: SUV_{mean,60 min} 0.94 ($n = 2$) in PDGFR β and 1.04 ± 0.14 ($n = 4$; $P < 0.01$) in PDGFR $\alpha + \beta$. The effect of combination

Figure 1

In vitro cell uptake data for radiotracers. (A) Effect of 48-h imatinib (0.3 μ M) and vemurafenib (0.25 μ M) treatment on [¹⁸F]FDG uptake into PDGFR α (+) and PDGFR α (-) B-CPAP cells treated with PDGF-AA (50 ng/mL). (B) Effect of 48-h long imatinib (0.3 μ M) treatment on [¹⁸F]FDG uptake into WT BRAF TPC-1 cells. Data shown as % radioactivity/mg protein and as mean $\pm$ S.E.M. from three experiments performed as triplicates. ** $P < 0.01$ and *** $P < 0.001$.

A

 $[^{18}\text{F}]\text{FDG}$

C

Figure 2

PET imaging using $[^{18}\text{F}]\text{FDG}$ in NSG xenografts bearing PDGFR α (+; right side) and PDGFR α (-; left side) B-CPAP tumours. (A) Representative images as maximum intensity projection after 60 min post injection for control animals injected with saline only (left), imatinib-treated mice (50 mg/kg for 6 days; middle left), under combination therapy of imatinib/vemurafenib (30 mg/kg + 20 mg/kg; middle right) and vemurafenib (20 mg/kg) therapy alone (right). (B) Time-activity curves (TACs) over 60 min dynamic experiments for $[^{18}\text{F}]\text{FDG}$ tumour (PDGFR α (-) - (left) and PDGFR α (+) - (right)) as well as non-targeting muscle tissue uptake under the same therapy conditions as mentioned previously. (C) Effects of imatinib, vemurafenib and combination therapy imatinib/vemurafenib on SUV_{mean} values at 60 min p.i. Data shown as SUV_{mean} and mean $\pm$ s.e.m. from n experiments. ** $P < 0.01$ and *** $P < 0.001$ vs control.

therapy using vemurafenib (20 mg/kg daily) and imatinib (30 mg/kg daily) led to a similar significant reduction of $[^{18}\text{F}]\text{FDG}$ uptake: $\text{SUV}_{\text{mean},60\text{min}}$ 0.76 ± 0.20 ($n=5$; $P < 0.001$) in PDGFR β and 0.82 ± 0.12 ($n=5$; $P < 0.001$) in PDGFR α + β . Uptake of $[^{18}\text{F}]\text{FDG}$ into non-targeting reference muscle tissue was not influenced by any therapeutic condition at all (Fig. 2B). Thus, results on glucose uptake and metabolism in xenograft tumours *in vivo* were consistent with the effects measured in BCPAP cells *in vitro*. This may be related to altered pERK signalling with PDGFR blockade, as shown in Fig. 3. Imatinib disruption of PDGFR induces changes in ERK signalling with appearance of the 50-kDa ERK isoform, first characterized by Ben-David *et al.* (2006), in both PDGFR α and PDGFR α + β tumours. All xenografts clearly demonstrate this alternatively spliced ERK form (Ben-David *et al.* 2006). Metabolic remodelling and altered ERK signalling pathway was previously seen altered in PDGFR and AKT pathway function shown by

Ekpe-Adewuyi *et al.* (2016). Sabbatino *et al.* (2014) also demonstrates PDGFR α and ERK signalling that mediates response to BRAF inhibitors. The absence of BRAF, or BRAF blockade, prevents reprogramming of glucose metabolism in our preclinical tumour model.

Tyrosine kinase inhibitor therapy effect on tumour growth

$[^{18}\text{F}]\text{FDG}$ tumour uptake was also correlated to xenograft tumour growth in all differently treated groups. Due to the substantially increased growth rate for PDGFR α + β tumours, a shorter lead growth time was used compared to tumours with only PDGFR β . Figure 4 depicts the tumour sizes for PDGFR β and PDGFR α + β tumours for imatinib therapy alone (Fig. 4A), single vemurafenib therapy (Fig. 4B) and combination therapy of imatinib and vemurafenib (Fig. 3C) before and after 6 days of therapy, at which time the

Figure 3

Western blots for analysis of PDGFR β and PDGFR α + β expressing BCPAP tumour tissue samples from NSG mice receiving either 50 mg/kg imatinib for 6 days (+) or control vehicle injections (-).

PET experiments were performed. Imatinib therapy alone was not effective in reducing tumour growth in both (PDGFR β and α + β) therapy groups vs the control groups. However, single therapy using vemurafenib led to a tendency of tumour growth reduction, while combination therapy of imatinib and vemurafenib was strikingly effective at reducing tumour burdens in both PDGFR β and PDGFR α + β tumours by nearly 90%: $121 \pm 23 \text{ mm}^3$ (control group) vs $16 \pm 6 \text{ mm}^3$ ($P < 0.01$, therapy group) for PDGFR β only and $209 \pm 54 \text{ mm}^3$ (control group) vs $29 \pm 6 \text{ mm}^3$ ($n = 5$, $P < 0.01$, therapy group) for PDGFR α + β .

Kinetic modelling of [^{18}F]FDG tumour uptake

Kinetic modelling was used to analyse the uptake of [^{18}F]FDG into BCPAP tumours using a standard, reversible two compartment model. Figure 5 displays analysed factors k_1 to k_4 and the ratios $k_3/(k_2+k_3)$ as well as $K_1 \cdot k_3/(k_2+k_3)$ for both PDGFR β (Fig. 5A) and PDGFR α + β (Fig. 5B) tumours. K_1 and k_2 were found to be similar and not significantly changed by any therapeutic intervention through all analysed thyroid tumours except some slight increases only in PDGFR α + tumours under single vemurafenib therapy. These two factors, K_1 and k_2 , quantify tracer transport through the active cell membrane, mainly by GLUT1, resulting in radiotracer tissue uptake. Factor k_3 represents the hexokinase-II mediated phosphorylation rate and did increase significantly for both BCPAP tumours: from $0.08 \pm 0.01 \text{ 1/min}$ to $0.15 \pm 0.03 \text{ 1/min}$ ($P < 0.01$) for PDGFR α + β tumours. Conversely both, individual blockade

of BRAF with vemurafenib, and combination therapy, strongly reduced k_3 in PDGFR α + β tumours: for vemurafenib $0.05 \pm 0.01 \text{ 1/min}$ ($P < 0.092$) and for combination therapy $0.02 \pm 0.004 \text{ 1/min}$ ($P < 0.01$) vs 0.08 ± 0.01 in control tumours. While the dephosphorylation rate k_4 was not changed with imatinib, single therapy with vemurafenib as well as combination therapy with both drugs increased it significantly from $0.013 \pm 0.001 \text{ 1/min}$ to $0.028 \pm 0.006 \text{ 1/min}$ ($P < 0.01$; vemurafenib alone) and $0.046 \pm 0.002 \text{ 1/min}$ ($P < 0.01$; combination therapy) in PDGFR α + tumours. Taken together, imatinib led to increased phosphorylation, while vemurafenib reduced phosphorylation and increased de-phosphorylation of [^{18}F]FDG. The ratio $k_3/(k_2+k_3)$ quantifies the fraction of radiotracer being shunted from the first into the second compartment phosphorylation, and this was increased under imatinib therapy in xenograft tumours from $0.29 \pm 0.02 \text{ 1/min}$ ($n = 13$) to $0.51 \pm 0.08 \text{ 1/min}$ ($P < 0.01$), but was strongly reduced by single therapy of vemurafenib resulting in $0.14 \pm 0.02 \text{ 1/min}$ ($n = 4$; $P < 0.05$) or combination therapy to $0.17 \pm 0.03 \text{ 1/min}$ ($n = 5$; $P < 0.01$),

Figure 4

Effect of therapeutic interventions on tumour sizes under the different conditions before therapy (control bars) and after 6 days of therapy (as indicated). (A) Single imatinib therapy (50 mg/kg), (B) single vemurafenib therapy (20 mg/kg) and (C) combination therapy (30 mg/kg imatinib + 20 mg/kg vemurafenib). Data shown as SUV_{mean} and mean $\pm$ s.e.m. from n experiments. ** $P < 0.01$ and *** $P < 0.001$.

Figure 5

Apparent tumor kinetic parameters K_1 to k_4 and ratios $k_3/(k_2+k_3)$ and $K_1 \cdot k_3/(k_2+k_3)$ for $[^{18}\text{F}]\text{FDG}$ tumor uptake as derived from a two-compartment model. Effects of single therapy with imatinib (red) or vemurafenib (blue) and combination therapy of imatinib/vemurafenib (green) are shown. Top, PDGFR α (-) B-CAP tumours; Bottom, PDGFR α (+) B-CAP tumours. Data shown as mean $\pm$ s.e.m. from n experiments. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs control.

respectively. The quotient $K_1 \cdot k_3/(k_2+k_3)$ then represents the absolute 'trapping' (phosphorylation) rate (mL/min per mL tissue), which was increased in the presence of imatinib, but significantly decreased when vemurafenib was added in a single or combination therapy (see also Fig. 2). Overall, there was no pronounced effect of PDGFR α expression on the $[^{18}\text{F}]\text{FDG}$ tracer kinetic profile *in vivo*.

GLUT1 and hexokinase II protein expression

Immunohistochemistry revealed a baseline expression of GLUT1 (Fig. 6) and hexokinase-2 (HK-II) in untreated

tumours (Fig. 7). Quantified protein positive staining showed single imatinib treatment led to a significant increase of GLUT1 expression (Fig. 6) *in vivo*: from $39.2 \pm 3.8\%$ to $61.2 \pm 1.4\%$ ($n=3$; $P < 0.01$) in PDGFR α tumours. Combination therapy with vemurafenib resulted in reduced GLUT1 expression which was closer to the control tumour levels: $50.0 \pm 0.8\%$ ($n=3$; n.s. vs controls). In contrast, hexokinase-2 protein expression (Fig. 7) tended to increase by single imatinib therapy but was significantly reduced during combination therapy with vemurafenib: $56.7 \pm 4.6\%$ in controls vs $67.6 \pm 9.4\%$ (imatinib; $n=3$; n.s.) and $33.6 \pm 4.9\%$ (imatinib+vemurafenib; $n=3$; $P < 0.05$) all

Figure 6

Representative immunohistochemical staining of GLUT1 in PDGFR α (-) and PDGFR α (+) tumour slices under control (left), single imatinib therapy (50 mg/kg for 6 days; middle) and combination therapy (30 mg/kg imatinib and 20 mg/kg vemurafenib for 6 days; right) conditions. (left) Original magnification of the entire tumor slice, $\times 5$, and enlarged areas at higher power, $\times 40$ from the same sample slices; (right) Quantification of positive GLUT1 immunohistochemical staining. Data as mean $\pm$ s.e.m. from $n=3$ slices, * $P < 0.05$, ** $P < 0.01$.

Figure 7

Representative immunohistochemical staining of hexokinase-2 in PDGFR α (-) and PDGFR α (+) tumour slices under control (left), single imatinib therapy (50 mg/kg for 6 days; middle) and combination therapy (30 mg/kg imatinib and 20 mg/kg vemurafenib for 6 days; right) conditions. (left) Original magnification of the entire tumor slice, $\times 5$, and enlarged areas at higher power, $\times 40$ from the same sample slices; (right) Quantification of positive hexokinase-2 (HK-II) immunohistochemical staining. Data as mean $\pm$ s.e.m. from $n = 3$ slices, * $P < 0.05$, ** $P < 0.01$.

in PDGFR β tumours, respectively. Protein expression data were consistent with our metabolic modelling analysis. Imatinib therapy alone increased uptake and trapping of glucose derivative [^{18}F]FDG. In contrast, combination therapy with vemurafenib led to a strong reduction of metabolic trapping indicating that reduced expression of hexokinase-2 was responsible for that. However, PDGFR α protein expression did not influence GLUT1 and hexokinase-2 expression.

Discussion

The present results reveal that the BRAF mutation enables metabolic re-modelling in thyroid cancer xenografts. The main findings are: (1) Tyrosine kinase receptor blockade of PDGFR with imatinib induced a strong increase in metabolic energy demand, possibly through the ERK pathway, as measured with the radioactive glucose derivative [^{18}F]FDG; (2) vemurafenib, targeting BRAF $^{\text{V600E}}$, leads to a significant reduction in metabolic glucose demand in the same cell model; (3) *in vivo* imatinib therapy induced a strong increase in metabolic glucose demand of BCPAP tumours as measured with [^{18}F]FDG, while single vemurafenib therapy resulted in the opposite, a significant reduction of [^{18}F]FDG uptake; (4) when imatinib and vemurafenib were combined also a strong reduction of energy metabolism and dramatic decreases in xenograft tumor growth occurred; and (5) the effect of imatinib on BCPAP energy metabolism maybe due to stronger upregulation of hexokinase-2 enzyme rather than GLUT1 transporters as detected with immunohistochemistry and analyzed with kinetic modelling.

PDGFR-targeting tyrosine kinase inhibitor imatinib binds both PDGFR α and PDGFR β (Valerio *et al.* 2017). Imatinib treatment increased [^{18}F]FDG uptake in both PDGFR α positive and negative cell lines significantly. PDGFR α expression does not appear to play any additional role for imatinib-induced effects on glycolytic energy metabolism in BCPAP cells. By blocking PDGFR and/or c-Kit in BCPAP cells, the glycolysis pathway seems to be upregulated. This is contrary to reports in other studies using imatinib as chemotherapeutic drug for gastrointestinal stromal tumors (GISTs) (Van den Abbeele *et al.* 2012). A comprehensive review of clinical studies confirmed that imatinib therapy initially reduces [^{18}F]FDG uptake by up to 40% in GIST tumours; however, after further c-KIT mutations under long-term therapeutic observations, this is abrogated (Treglia *et al.* 2012). Vemurafenib significantly reduced [^{18}F]FDG uptake in both PDGFR β and PDGFR α + β cell lines. Vemurafenib blocks c-Kit/PDGFR downstream target BRAF $^{\text{V600E}}$ mutation directly. This indicates that c-Kit/PDGFR blocking with imatinib induces the increased energy metabolism through BRAF $^{\text{V600E}}$ mutated BCPAP cells, while blockade of BRAF $^{\text{V600E}}$ leads to downregulation of glucose uptake and metabolism in these cells. This metabolic remodelling has been seen before in melanoma and colorectal cancer cells (Hutton *et al.* 2016, Lee *et al.* 2016). *In vitro* control experiments with imatinib in TPC1 cells, expressing WT BRAF, did not lead to any increased [^{18}F]FDG uptake. Therefore, it appears that the BRAF $^{\text{V600E}}$ mutation is indeed a key target for glycolytic metabolism in BCPAP thyroid cancer cells. BRAF $^{\text{V600E}}$ mutation and [^{18}F]FDG avidity have already been linked in PTC (Santhanam *et al.* 2018). These studies support the

conclusion that metabolic remodelling in thyroid cancer as a consequence of chemotherapy may be monitored using non-invasive [^{18}F]FDG-PET.

BRAF^{V600E} mutation and [^{18}F]FDG avidity have already been linked in PTC (Nagarajah *et al.* 2015, Ferretta *et al.* 2016, Hutton *et al.* 2016, Lee *et al.* 2016). Lee *et al.* have shown that thyroid cancer patients with BRAF^{V600E} mutation showed higher [^{18}F]FDG uptake than thyroid cancers without it (Lee *et al.* 2016). Yoon *et al.* confirmed these findings and reported that ~65% of PTC patients carried the BRAF^{V600E} mutation (Yoon *et al.* 2016). And a recent review by Ciavardelli *et al.* summarized numerous clinical studies revealing a link between GLUT1 transporters and hexokinase-2 among other downstream targets with BRAF^{V600E} mutation in thyroid cancer (Ciavardelli *et al.* 2017). Analysis of single therapy with imatinib and combination therapy with imatinib and vemurafenib in B-CPAP xenograft model *in vivo* resulted in similar effects as detected *in vitro*. Glucose energy metabolism was turned on with c-Kit/PDGFR blockade using imatinib, while addition of BRAF^{V600E} mutation targeting vemurafenib resulted in a strong downregulation of energy metabolism as detected with [^{18}F]FDG PET. Clinical studies in melanoma patients have described complete metabolic response of vemurafenib on [^{18}F]FDG uptake (Pascal *et al.* 2018). Parmenter *et al.* have shown that BRAF inhibition in BRAF-mutant melanoma is mediated by a network of transcriptional regulators of glycolysis including hypoxia-inducible factor 1 α (HIF-1 α) following downstream targets GLUT1 and 3 as well hexokinase-2 (Parmenter *et al.* 2014). This confirms that the pro-oncogene BRAF^{V600E} regulates downstream glucose energy metabolism as can be assessed with [^{18}F]FDG functional analysis. Reports in BCPAP cell line and clinical specimens have also linked pyruvate kinase M2, a glucose metabolism protein, with the BRAF^{V600E} mutation (Feng *et al.* 2013).

In contrast to previous studies, the present work demonstrates that it is likely hexokinase II upregulation that confers this tumour phenotype and not changes in GLUT 1 expression (Yoon *et al.* 2016). Baseline levels of GLUT1 expression may vary based on BRAF^{V600E}; however, compartmental analysis demonstrates that the intracellular trapping levels in BCPAP tumours are governed by the phosphorylation rate and hexokinase-2 activity. Imatinib therapy alone led to an increase in phosphorylation as expressed in the ratio $k_3/(k_2+k_3)$, as well as increase in the absolute 'trapping' rate $K_1 \cdot k_3/(k_2+k_3)$, pointing to functional upregulation of hexokinase-2 enzyme activity. Single vemurafenib therapy

or in combination with imatinib both led to the opposite: reduced phosphorylation rates and a strong suppression of 'trapping'. Kinetic analysis confirmed no significant change of K_1 and k_2 under any treatment regimen, indicating that [^{18}F]FDG uptake, facilitated mainly through GLUT1 transporters, may not be substantially altered by blocking the BRAF^{V600E} mutation. Moreover, the immunohistochemistry clearly demonstrates in the xenograft tumour samples that a significantly increased expression of hexokinase-2 occurs. These results therefore point towards the regulation of hexokinase-2, rather than GLUT1 transporter, through the thyroid mutated oncogene BRAF^{V600E}. Blocking of PDGFR α + β leads to changes in ERK and, further downstream, the increase of hexokinase-2 expression resulting in higher metabolic glucose demand as measured with [^{18}F]FDG. Changes in pERK levels and altered ERK isoforms have been documented previously with our work on changes in PDGFRA signalling and blockade (Ekpe *et al.* 2016), as well as by Ben-David *et al.* (2006) and Sabbatino *et al.* (2014). The relationship between ERK signalling and [^{18}F]FDG uptake has also been well documented in clinical analysis of MEK inhibitors in different metastatic solid tumours, whereby decreased ERK activity follows a reduction in [^{18}F]FDG uptake (Kraeber-Bodéré *et al.* 2012).

[^{18}F]FDG-PET functional analysis of BRAF^{V600E} thyroid carcinomas may be a useful adjunct to directed therapy for advanced disease. Clinical studies in melanoma patients have described complete metabolic response of vemurafenib based on [^{18}F]FDG uptake (Pascal *et al.* 2018). Parmenter *et al.* have shown that BRAF inhibition in BRAF-mutant melanoma is mediated by a network of transcriptional regulators of glycolysis including hypoxia-inducible factor 1 α (HIF-1 α) following downstream targets GLUT1 and hexokinase-2, similar to what we observed in our xenografts (Parmenter *et al.* 2014). Inhibition of BRAF^{V600E} strongly reduces tumour size and uptake of glucose derivative [^{18}F]FDG, while PDGFR inhibition by imatinib paradoxically increases glycolysis based on upregulation of hexokinase-2 protein levels. While PDGFR α expression correlates with the metastatic potential in PTC, it appears that it is not a driver for glycolysis in thyroid cancer cells. However, it is important to note that Sabbatino *et al.* discovered that PDGFR α up-regulation, MAPK reactivation and PI3K/AKT activation lead to BRAF inhibitor resistance in melanoma cells (Sabbatino *et al.* 2014). The authors suggested that monitoring patients for early PDGFR α up-regulation will facilitate the identification of those who may benefit from the treatment with BRAF inhibitor in combination with

clinically approved PDGFR α inhibitors. It remains to be elucidated in clinical studies if this would be also the case for the therapy of PTC patients using the various multi-targeting TKI developed over the past two decades.

Short-term imatinib therapy of 6 days induced changes in GLUT 1 and hexokinase-2 protein expression in BCPAP tumours *in vivo*. Upregulation of protein expression was more pronounced for GLUT1 than hexokinase-2. This finding points to an increased glucose metabolism in BCPAP thyroid cancer cells, which is contradictory to studies in other cancers where TKI therapy led to a reduced energy metabolism detected by [^{18}F]FDG (Vercellino *et al.* 2009, Ueno *et al.* 2012, Van den Abbeele *et al.* 2012, Ma *et al.* 2017). Short-term imatinib therapy in GIST leads to reduction of [^{18}F]FDG uptake and retention (Prenen *et al.* 2006, Revheim *et al.* 2011). However, GIST cells can adopt and change their metabolic energy supply leading to imatinib resistance (Vitiello *et al.* 2018). Comparing to studies using TKI sorafenib, which also targets c-Kit and PDGFR but in addition also VEGFR, glucose metabolism is also strongly reduced in colorectal cancer model HCT116 (Tseng *et al.* 2017). Similar to sorafenib, TKI sunitinib, which targets similar proteins except Raf, also leads to reduction of [^{18}F]FDG uptake after 6 days treatment in this case in breast cancer (Thézé *et al.* 2015). However, GLUT1 is upregulated under sunitinib therapy. Thus targeting VEGFR, c-Kit, PDGFR and RET, as well as BRAF, may lead to GLUT 1 upregulation and a downregulation of the overall glucose energy metabolism, as shown here. Only increases in hexokinase-2 expression as detected with imatinib resulted in a 'switch-on' of glucose consumption in BRAF^{V600E} mutated BCPAP cells confirming BRAF^{V600E} as the key-oncogene for energy metabolism as discussed previously.

Conclusions

The pro-oncogene BRAF^{V600E} is the glycolytic driver in BCPAP thyroid cancer cells. Inhibition of BRAF^{V600E} strongly reduces tumour size and uptake of glucose derivative [^{18}F]FDG, while PDGFR β inhibition by imatinib paradoxically increases glycolysis based on upregulation of GLUT1 protein levels. Imatinib-induced metabolic re-modelling is mediated by upregulation of hexokinase II through BRAF^{V600E}. Blocking of PDGFR α + β results in upregulation of downstream p-ERK which triggers the increased metabolic glucose demand in BCPAP cells and tumours. Additional PDGFR α expression in BCPAP cells and tumours did not change glycolytic metabolism

in BCPAP cells and tumours. As PDGFR α expression correlates with the metastatic potential in PTC, it is not a driver for glycolysis in BCPAP cells. [^{18}F]FDG-PET could be used to monitor the effectiveness of therapy in thyroid cancer patients and potentially treatment resistance. It remains to be proven if non-invasive determined PDGFR α expression in PTC patients would result in the selection of a beneficial therapy regimen using a BRAF inhibitor alone or in combination with clinically approved PDGFR α inhibitor to substantially decrease tumor burden for patients with metastatic disease.

Declaration of interest

Todd McMullen holds a patent entitled 'Methods, kits, and systems for treatment of metastatic papillary thyroid cancer' (US9897607B2, Feb 20, 2018) and is an applicant for the patent 'Targeted therapy to restore radioactive iodine transport in thyroid cancer' (WO2015166355A3). The other authors have nothing to disclose.

Funding

This work was supported by the Canadian Institute of Health Research (CIHR).

Acknowledgements

The authors thank Angela Westover, Kristan Nagy-Colombo and Jeff McPherson for the production of GMP quality [^{18}F]FDG and Ali Akbari for the generous production of [^{18}F]FLT. The authors thank Dan McGinn (Vivarium of the Cross Cancer Institute, Edmonton, AB, Canada) for supporting the animal work. Michael Wagner is grateful for a Canadian Institute of Health Research (CIHR) postdoctoral fellowship. The authors also gratefully acknowledge the Dianne and Irving Kipnes Foundation for supporting this work.

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Received in final form 18 June 2020

Accepted 25 June 2020

Accepted Manuscript published online 25 June 2020