Title
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Journal
Discover Oncology, 7(3)

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Publication Date
2016-06-01

DOI
10.1007/s12672-016-0253-6

Peer reviewed

HORM CANC (2016) 7:188–195
DOI 10.1007/s12672-016-0253-6

ORIGINAL PAPER

pAKT Expression and Response to Sorafenib in Differentiated Thyroid Cancer

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Received: 20 December 2015 / Accepted: 2 February 2016 / Published online: 18 March 2016

Abstract Sorafenib has an antitumor activity in patients with radioactive iodine-refractory differentiated thyroid carcinoma (RAIR-DTC). Prior research has implicated signaling through the MAPK and AKT/PI3K pathways in the progression of DTC. To assess whether the activity of these pathways is predictive of response to sorafenib, we retrospectively studied molecular tumor markers from these two pathways from a phase 2 study of sorafenib in RAIR-DTC. Tumor samples from 40 of 53 DTC subjects obtained prior to initiation of sorafenib were immunostained with DAB-labeled antibodies to phospho-AKT (pAKT), phospho-ERK (pERK), and phospho-S6 (pS6). BRAFV600E genetic mutation analysis was performed on all samples. Expression levels and mutational status were compared to response and progression-free survival (PFS) for each patient. Low tumor expression of nuclear pAKT was associated with partial response to sorafenib ($p < 0.01$). Patients with nuclear pAKT expression that was below the median for our sample were more than three times as likely to have a partial response as patients with equal to or above median expression. There was no correlation between tumor expression of nuclear pERK or pS6 and response. Endothelial cell and pericyte expression of pERK, pAKT, and pS6 were not predictive of response. There was no correlation between BRAFV600E mutation status and partial

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response. No correlation was observed between either the expression of pAKT, pERK, or pS6, or the presence of the BRAFV600E mutation, and PFS. In conclusion, lower tumor expression of nuclear pAKT was associated with higher rate of response to sorafenib. This observation justifies evaluation of combination therapy with sorafenib and an inhibitor of the PI3K/AKT signaling pathway in RAIR-DTC.

Introduction

Thyroid cancer is the most common endocrine malignancy, with more than 60,000 new cases and 1850 deaths from this disease annually in the USA alone. Differentiated thyroid cancer (DTC) accounts for 80–90 % of all thyroid carcinomas and includes papillary and follicular subtypes [1]. First-line treatment for DTC is based on surgical resection as well as radioactive iodine (I-131), followed by thyroid hormone suppression [2]. While overall prognosis of DTC is usually excellent with a 10-year survival of 85 %, some patients with DTC develop a more aggressive form of the disease with distant metastasis and radioactive iodine-refractory DTC (RAIR-DTC).

Until recently, treatment options for patients with progressive, metastatic or unresectable, radioactive iodine-resistant advanced thyroid cancer were limited. Only approximately 25 % of such patients respond to conventional chemotherapy, and the historical median survival of the doxorubicin-based treatment regimens was approximately 8 months [3, 4]. However, in 2013, the US Food and Drug Administration (FDA) approved the multikinase inhibitor sorafenib for the treatment of metastatic DTC after a phase III registrational study confirmed the efficacy of sorafenib in patients with progressing RAIR-DTC [5]. Sorafenib is an inhibitor of multiple intracellular (c-CRAF, BRAF) and cell surface kinases [including vascular endothelial growth factor receptor 2 (VEGFR2), platelet-derived growth factor receptor (PDGFR), FLT3, rearranged in transformation (RET), and c-Kit] [6]. Serial biopsies from patients with DTC treated with sorafenib have confirmed that the drug has an antiangiogenic activity and that it inhibits the RAS-RAF kinase signaling pathway [7]. Therefore, the efficacy of sorafenib activity in DTC is thought to be related in part to direct effects on the tumor through RAF and other cell signaling pathways, as well as effects on the tumor environment by inhibiting VEGFR2 and PDGFR signaling.

Accelerated approval of sorafenib in metastatic DTC was based on clinical evidence that sorafenib significantly improved progression-free survival compared with placebo. However, clinical response to sorafenib is variable, and all patients eventually become resistant to therapy [8]. At present, data on possible predictive molecular biomarkers for response to sorafenib are limited, and no predictive molecular biomarkers have entered into clinical practice. Recently, a number of genetic alterations have been discovered and characterized in thyroid cancer [9–11]. Many of these mutations lead to the activation of the mitogen-activated protein kinase (MAPK) pathway, or the PI3K/AKT signaling pathway, and therefore, activity of these pathways has been implicated in the pathogenesis and progression of DTC. To assess the clinical significance of activity in these two pathways, we measured levels of expression of p-MAPK (phospho-ERK (pERK) in mammalian cells) of the MAP-kinase signaling pathway and phospho-AKT (pAKT) and phospho-S6 (pS6) of the PI3K signaling pathway and assessed the correlation of the expression level of these biomarkers with subsequent clinical responses to sorafenib therapy. We also studied whether the presence or absence of the BRAF T1799A (*BRAFV600E*) mutation, an activating single amino acid substitution of the BRAF kinase that is described to occur in multiple neoplasms including DTC, is predictive of response to sorafenib therapy.

Methods

Patients

Tumor pathology and corresponding clinical data were obtained from patients entered in a phase 2 study of sorafenib in advanced thyroid cancer between 2006 and 2009 (Clinical trials NCT00654238) [12]. Written and verbal informed consent to participate in the study was obtained from all study participants at the time of enrollment. This research was performed in accordance with the Declaration of Helsinki, and the study protocol was approved by the Institutional Review Board at the University of Pennsylvania.

Eligible patients were at least 18 years old and had metastatic or unresectable thyroid carcinoma with evidence of disease progression in the year before initiation of treatment. Other eligibility criteria included Eastern Cooperative Oncology Group (ECOG) performance status of less than 2 and life expectancy more than 3 months. Patients were ineligible if they had prior exposure to a RAS-RAF pathway inhibitor (including but not limited to EGFR inhibitors or MEK inhibitors). Sorafenib was administered at an initial dose of 400 mg orally twice a day, although dose reductions and interruptions were sometimes necessary to manage side effects. Doses were reduced in 18 of 40 patients (45 %) to control symptoms. For patients requiring dose reductions, sorafenib was initially decreased by 25 % (to a total daily dose of 600 mg), and six patients (15 %) required up to 50 % dose reductions (400 mg total daily). Clinical response to sorafenib therapy was assessed based on the findings on computed tomography (CT) or magnetic resonance imaging (MRI) using Response Evaluation Criteria in Solid Tumors (RECIST 1.0)

[13]. All responses were confirmed by a study-designated radiologist.

Of patients originally entered into this phase 2 trial of sorafenib, only patients who received at least 1 month of drug and were evaluable for response were included in this analysis. We also excluded patients with a pathologic diagnosis of medullary thyroid cancer or anaplastic thyroid cancer. Of the remaining 53 patients, we were able to obtain sufficient tissue in time for biomarker evaluation in 40 patients. In all cases, the most recent tumor sample from each patient obtained prior to initiation of sorafenib was used for tumor marker analysis. Of the 40 tumor samples, 24 samples were obtained from the primary tumor site, 4 samples were from regional neck lymph nodes, and the remaining 12 samples were obtained from distant metastasis (most often the lungs).

Immunostaining

Immunostaining was overseen by a single operator who was blinded to the clinical responses of the subjects. All slides were processed together in parallel. Paraffin-embedded tissue sections were heated to 70 °C, then deparaffinized in xylene, and rehydrated in graded ethanols. Antigen retrieval was performed by submerging slides in the antigen retrieval solution (10 mM sodium citrate buffer at pH 6.0) and boiled at 95 °C for 25 min. Endogenous peroxidase activity was blocked with 3 % H_2O_2. Subsequently, slides were blocked for 1 h in blocking buffer (PBST + 10 % goat serum + 1 % BSA) and then incubated in the primary antibody overnight at 4 °C, followed by a 30-min incubation with biotinylated goat anti-rabbit IgG at a 1:200 dilution. Primary antibodies used were pERK (Cell Signaling 4370, Rabbit, 1:100), pAKT (Cell Signaling 3787, Rabbit, 1:50), and pS6 (Cell Signaling 4858, Rabbit, 1:50). Following the incubation with the secondary antibody, slides were then incubated with the ABC reagent (Vector Laboratories, Burlingame, CA) for 30 min and antibodies were labeled with DAB solution.

One of the 40 samples failed in the staining for nuclear pAKT and was excluded from analysis for this tumor marker. This was a sample from a patient with PTC who achieved a partial response (PR) on sorafenib. Additionally, tumor endothelial staining could not be performed on two samples for pERK, two samples for pAKT, and four samples for pS6. *BRAFV600E* genetic mutation analysis was performed on all samples using a mass spectrometry genotyping assay (Sequenom, San Diego, CA) and confirmed with the Sanger sequencing method.

Multispectral Imaging Analysis

Slides were examined using a Vectra whole slide multispectral imaging robot (Perkin Elmer, Woburn, MA) equipped with planapochromatic lenses. Images of each immunohistochemical study were imaged at ×20 through a liquid crystal filter using the Inform Multispectral Imaging System (Perkin Elmer, Woburn, MA). This imaging system is based on a tunable liquid crystal technology that is linked to a charge-coupled device (CCD) camera and a personal computer (PC). The MSI system was used at full chip resolution without data binning. Spectral data were acquired from 420 to 720 nm in 10-nm increments. Spectral unmixing was accomplished by Nuance software v1.42 and pure spectral libraries of individual chromogens (slides stained with only DAB or hematoxylin). Nonspecific background staining was subtracted from each image individually.

Inform image analysis software v2.0 (Perkin Elmer, Woburn, MA) was used to automatically segment individual images into tumor regions and non-tumor regions using a train by example approach where an expert pathologist segmented a couple of areas (tumor versus non-tumor regions) on each image; these were then used by the software's tissue-finding algorithm to classify the rest of the image into tumor and non-tumor regions. Following tissue segmentation, the resulting tumor regions were then further analyzed using the cell segmentation algorithm in Inform which identifies cell nuclei within a tumor region. The optical density of the DAB for each identified nucleus is then scored and output on a per cell basis as well as binned into 4 categorical bins (0–3+) based on defined thresholds. The results of 4–6 fields of view for each stain on each tumor were then averaged (allows capture of 1000–2000 cell events) to produce a global measurement of tumor protein expression. Representative immunohistochemical staining for pERK, pAKT, and pS6 are demonstrated in Fig. 1.

Endothelial cells were scored manually after the Inform tissue segmentation tool was found to be unable to accurately and reliably segment endothelial cells. Manual scoring was performed by a single experienced operator blinded to the clinical response of the patient. Scoring was performed by examining the tumor slide and scoring the intensity of DAB and abundance of endothelial cell nuclei with DAB staining. The intensity and abundance were then combined into a 4-bin scoring (none = 0, low = 1, moderate = 2 or high = 3) scoring system. A formal H score was not employed as the number of endothelial cells in each field is small (10–60 endothelial cell nuclei per field of view) making accurate estimates of percent staining challenging.

Statistical Analysis

BRAF mutation status and protein expression levels of nuclear pERK, pAKT, and pS6 in tumor and endothelial tissue were correlated with objective tumor response to sorafenib. Using an analysis of variance (ANOVA), we compared protein expression levels of patients who achieved a partial response to sorafenib to those who did not experience a partial response. BRAF mutation status was correlated with clinical response to

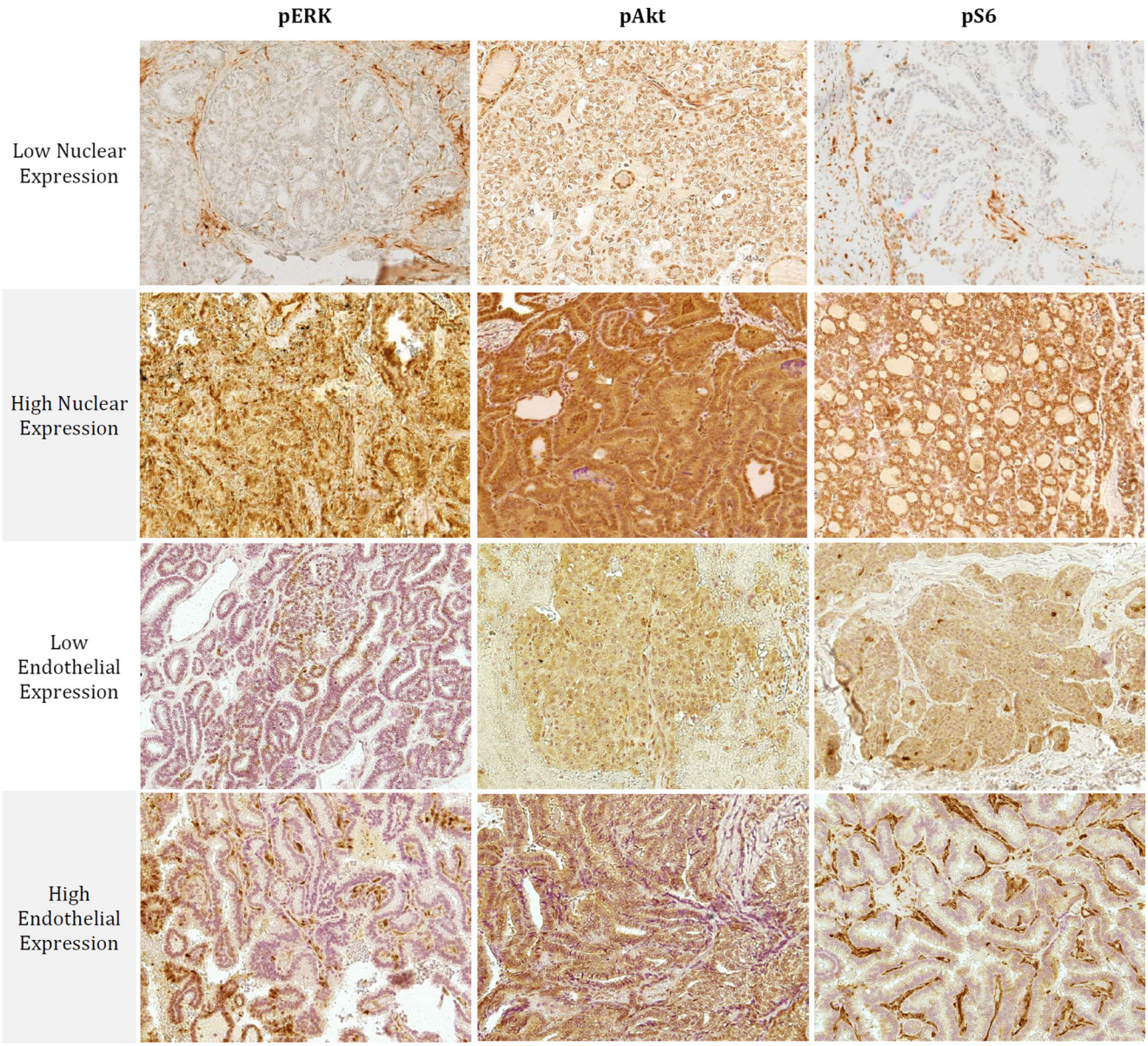

Fig. 1 Representative immunohistochemical staining for pERK, pAKT, and pS6 demonstrating higher or lower expression of each protein in tumor and endothelial cells

sorafenib using a χ^2 analysis. Correlations of pERK, pAKT, and pS6 expression with progression-free survival (PFS) (defined as time in months from initiation of drug therapy to disease progression or death, whichever occurred first) were performed using linear regression analyses. All statistical analyses were performed using JMP Pro 11 (SAS Institute Inc., Cary, NC). p values of less than 0.05 were considered statistically significant, and all statistical tests were two sided.

Results

Biomarker analysis was performed for a total of 40 RAIR-DTC patients on sorafenib therapy. Characteristics of the study group are displayed in Table 1. The majority of patients in our study group had papillary thyroid cancer (PTC; $n=22$) or follicular thyroid cancer (FTC; $n=14$), whereas a small number of subjects had poorly differentiated thyroid cancer (PDTC; $n=4$). Across the entire study group, 17 patients (42.5 %) achieved a partial response (PR), 21 (52.5 %) achieved stable disease (SD), and two (5 %) had disease progression as a best response to sorafenib therapy.

We found no association between nuclear pAKT, pERK, and pS6 expression in tumor or endothelium and age or sex. Similarly, there was no significant association between *BRAFV600E* mutation status and age or sex, consistent with prior analyses in larger patient samples of the relationship between the *BRAFV600E* mutation status and

Table 1 Baseline characteristics of the patient study group and clinical responses to sorafenib

Age	64 (10.4)
Sex	
Male	19 (47.5 %)
Female	21 (52.5 %)
BMI	26.8 (4.6)
Tumor pathology	
FTC	14 (35 %)
PTC	22 (55 %)
PD	4 (10 %)
BRAF V600E mutation status	
Pos	9 (22.5 %)
Neg	31 (77.5 %)
Best response	
CR	0 (0 %)
PR	17 (42.5 %)
SD	21 (52.4 %)
PD	2 (5 %)
Progression-free survival (months)	19 (12.8)

Values given are mean (standard deviation) or number (percentage)

clinicopathologic factors in thyroid cancer [14]. Additionally, *BRAFV600E* mutation status was not correlated with nuclear or endothelial expression of pAKT, pERK, and pS6.

Expression of Nuclear pAKT, pERK, and pS6 in Tumor Cells

Expression of nuclear pAKT was significantly and inversely associated with a partial response to sorafenib ($p < 0.01$, Fig. 2a). The majority (12/19) of subjects with nuclear pAKT expression that was lower than the median for our sample experienced a PR on sorafenib. Of the 10 subjects with the lowest nuclear pAKT expression in our cohort, seven achieved a PR. By contrast, only four of 22 subjects with nuclear pAKT expression that was equal to or above the median for our sample experienced a PR. Furthermore, the only two patients in our cohort who had disease progression as a best response had relatively high expression of nuclear pAKT. The expression of nuclear pAKT was similar for the subset of patients with papillary thyroid cancer as with the larger cohort. As with the larger sample, tumor expression of nuclear pAKT was inversely associated with a PR within the subset of patients with papillary thyroid cancer ($p < 0.05$).

While tumor expression of nuclear pERK and nuclear pS6 also appeared to inversely correlate with a partial response to sorafenib, this association was not statistically significant (all $p > 0.05$) (Fig. 2b, c). We repeated these analyses for the subset of patients with papillary thyroid cancer and again found no association between clinical response to sorafenib and tumor expression of nuclear pERK or pS6 (all $p > 0.05$). We separately examined correlations between pAKT, pERK, and pS6 expression in tumor and PFS on sorafenib. There was no association between tumor expression of nuclear pAKT, pERK, and pS6 with PFS on sorafenib across the entire sample, or within the subset of patients with papillary thyroid cancer (all $p > 0.05$).

Endothelial Cell and Pericyte Expression of pAKT, pERK, and pS6

There was no association between clinical response to sorafenib and endothelial cell and pericyte expression of pAKT, pERK, and pS6 (all $p > 0.05$). We repeated these analyses for the subset of patients with papillary thyroid cancer and again found no association between clinical response to sorafenib and endothelial expression of any of the candidate proteins (all $p > 0.05$). Across the entire patient sample and within the subset of patients with papillary thyroid cancer, there was no association between endothelial cell and pericyte expression of pAKT, pERK, or pS6 and PFS on sorafenib (all $p > 0.05$).

BRAFV600E Mutation Status

A *BRAFV600E* mutation was identified in nine patients (22.5 % of our study sample). Across the entire cohort, the presence or absence of a BRAFV600E mutation did not confer a significant difference in overall tumor response ($p > 0.05$, Fig. 3). In the BRAF mutant group, one patient (11 %) had PD, three patients (33 %) had SD, and five patients (56 %) had PR. In the BRAF wild-type group, one patient (3 %) had PD, 18 (58 %) had SD, and 12 (39 %) had PR.

Consistent with previous reports (14–16), the *BRAFV600E* mutation was more common among subjects with PTC than FTC or PDTC. In our sample, 8 of our 22 subjects with papillary thyroid cancer had a *BRAFV600E* mutation, whereas only 1 of 18 subjects with FTC or PDTC had this mutation. Therefore, we separately examined whether a *BRAFV600E* mutation was predictive of partial response to sorafenib within the subset of patients in our cohort with papillary thyroid cancer ($n = 22$). In this subgroup analysis, we again found no significant difference in response rate by *BRAFV600E* mutation status ($p > 0.05$).

Discussion

In order to identify possible molecular biomarkers of response to sorafenib in patients with metastatic RAIR-DTC, we used quantitative immunohistochemistry to measure baseline expression of several candidate proteins in tumor cells and in surrounding endothelial cells prior to initiation of sorafenib and correlated these measurements with the patient's clinical

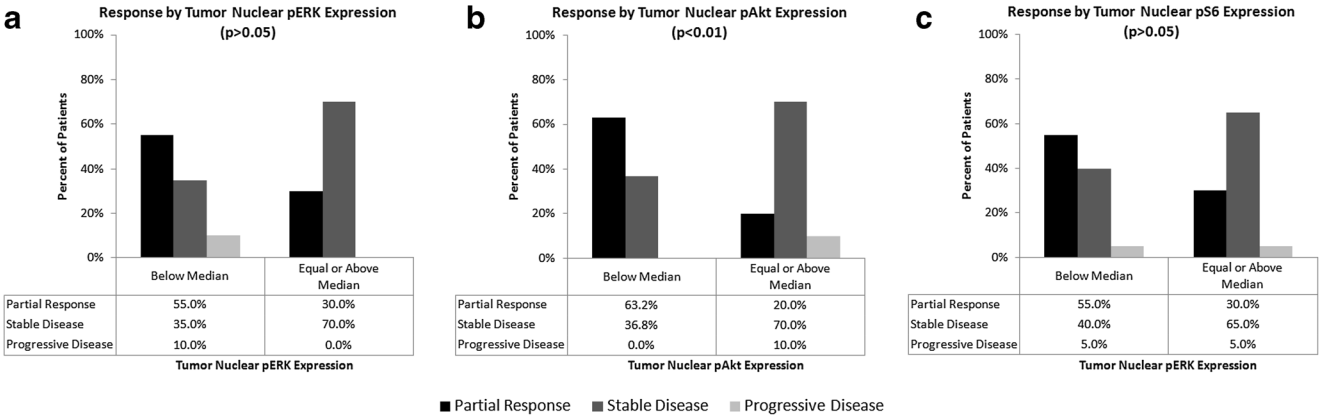

Fig. 2 Protein expression levels of nuclear pERK, pAKT, and pS6 in tumor and clinical response to sorafenib. Here, responses to sorafenib are stratified by whether protein expression levels for each candidate protein were equal to or above vs. below the numerical median for the entire sample. (**a**) Subjects with lower expression of nuclear pAKT were more likely to experience a partial response to sorafenib ($p < 0.01$). The majority of patients (63.2 %) with tumor nuclear pAKT expression that was below the median for our sample experienced a partial response to sorafenib, as compared to 20 % of subjects with tumors that had equal to or above the median pAKT expression. (**b** and **c**). There was no correlation between expression of pERK or pS6 and clinical response to sorafenib ($p > 0.05$)

response. We found that patients with thyroid tumors expressing lower levels of nuclear pAKT were more likely to achieve a partial response to sorafenib. There was no significant association between the remaining biomarkers we analyzed in tumor or endothelial cells and clinical response to sorafenib. To our knowledge, this is the first time that a molecular biomarker in pre-treatment biopsy samples has been identified to predict clinical response to sorafenib in the setting of a clinical trial for advanced thyroid cancer. In one retrospective series, a decrease of greater than 50 % in the thyroid cancer tumor markers CEA/calcitonin or thyroglobulin after initiation of sorafenib therapy was correlated with a partial response [15]. It is unclear if the decrease is due to death of tumor cells or decreased secretion of the markers or both, and larger series will be needed to determine the utility of this observation. However, in contrast to serum tumor markers on treatment, nuclear pAKT expression predicts for response and therefore may have clinical utility in weighing treatment options.

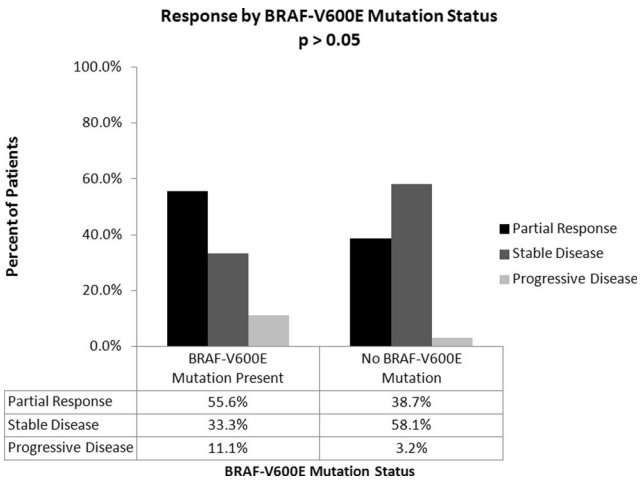

Fig. 3 Objective response rates to sorafenib were not statistically different among patients with and without a BRAF-V600E mutation ($p > 0.05$)

The PI3K/AKT signaling pathway has previously been described to play an important role in thyroid cancer tumorigenesis and progression [9–11]. This pathway regulates several important cellular functions such as cell growth, proliferation, glucose uptake, and cell survival. Several genetic alterations of this pathway have previously been described in thyroid cancer that could lead to increased activation, including amplification/copy gain of PIK3CA, PIK3CB, PDK1, and AKT [11]. Therefore, it is possible that increased pAKT expression in tumor cells is associated with a less robust response to sorafenib because it indicates PI3K/AKT signaling pathway activation downstream of VEGFR, where sorafenib may act. It is also noteworthy that expression of pS6 was not correlated with response to sorafenib, since both pS6 and pAKT belong to the same PI3K/AKT signaling pathway. Since phosphorylation of AKT occurs upstream of phosphorylation of S6 and has multiple downstream effectors, we speculate that functional resistance to sorafenib may be mediated by pAKT-dependent activity other than pS6. Our results differ from biomarker studies of sorafenib in other tumor types. For example, in hepatocellular carcinoma, increased baseline pERK expression is associated with a prolonged time to progression on sorafenib therapy [16]. In our study, there was no correlation between tumor pERK expression and PFS or response to sorafenib. However, this may be due to our small sample size and lack of power to detect an association and not necessarily due to differences in mechanisms between the two tumor types.

If lower expression of pAKT is confirmed in additional studies to be predictive of a higher likelihood of partial response to sorafenib, this could have several implications for the clinical management of thyroid cancer. First, analysis of nuclear pAKT could be used to identify patients who are most

likely to benefit from sorafenib therapy, while allowing patients less likely to attain a partial response to proceed to other therapeutic options or combination therapy, especially in situations where a response is clinically required. Second, our findings provide support for use of combination therapy for the management of advanced thyroid cancer. Since signaling through pAKT and the PI3K pathway has emerged in our study as a possible resistance mechanism for sorafenib, we hypothesize that the addition of an inhibitor of the PI3K/AKT signaling pathway may augment the observed clinical responses to sorafenib in some patients. We recently tested this hypothesis in a pilot phase 2 single-arm trial of combination use of the mammalian target of rapamycin (mTOR) inhibitor everolimus in addition to sorafenib in patients who have progressed on sorafenib alone [17]. In this trial, the addition of everolimus to sorafenib provided patients with a mean additional period of PFS of 13.9 months following progression on sorafenib alone. In addition to everolimus, several other inhibitors of the PI3K/AKT signaling pathway are approved or in clinical development. We believe that further investigation of everolimus and other inhibitors of the PI3K/AKT signaling pathway is warranted in patients with metastatic RAIR-DTC. At the time that this biomarker study was conceived, sorafenib was the only approved molecularly targeted therapy for metastatic RAIR-DTC. However, the multitargeted tyrosine kinase inhibitor lenvatinib recently received FDA approval for this indication [18]. Lenvatinib and sorafenib have many of the same molecular targets, and therefore, it is possible that pAKT will also be predictive of response to lenvatinib therapy and that the addition of everolimus with lenvatinib will also have clinical benefit. Additional research is needed to identify potential biomarkers of response to lenvatinib.

Notable strengths of this investigation included the use of a moderately sized and clinicopathologically diverse patient sample with well-documented clinical outcomes, the use of highly sensitive and specific DAB-labeled antibodies to stain all clinical samples processed in parallel, and the use of quantitative image analyses which significantly diminished intra-observer variability during the reading of the IHC. Relative weaknesses include selection biases inherent in our small cohort. Additionally, while cancers are inherently heterogeneous, only a single biopsy from each patient representing only a part of a single tumor was analyzed. However, sample heterogeneity would be expected to bias our results towards the null hypothesis. Because our analyses were exploratory in nature, we did not correct for multiple comparisons. In summary, this study demonstrates that in patients with RAIR-DTC, tumors expressing lower levels of pAKT are more likely to obtain a partial response from sorafenib therapy. If this association is confirmed in additional independent samples, pAKT could be used clinically. Finally, our results indicate the potential role for the addition of a PI3K/AKT signaling pathway inhibitor in the treatment of RAIR-DTC.

DTC, differentiated thyroid carcinoma; FDA, Food and Drug Administration; FTC, follicular thyroid cancer; MAPK, mitogen-activated protein kinase; PD, progressive disease; PDGFR, platelet-derived growth factor receptor; PDTC, poorly differentiated thyroid cancer; PR, partial response; PTC, papillary thyroid cancer; RAIR, radioactive iodine-refractory; RET, rearranged in transformation; SD, stable disease; VEGFR2, vascular endothelial growth factor receptor 2.

Acknowledgments This study was supported by the Templeton Family Cancer Research Fund, Liz and Geoff Watson Research Fund, Stanley and Lois Elkman Research Fund, REACT Thyroid Foundation, and the Damon Runyon Cancer Research Foundation (CI-25-05; M.S.B.).

Compliance with Ethical Standards Written and verbal informed consent to participate in the study was obtained from all study participants at the time of enrollment. This research was performed in accordance with the Declaration of Helsinki, and the study protocol was approved by the Institutional Review Board at the University of Pennsylvania.

Conflict of Interest Dr. Marcia S. Brose has received grant/research support and honoraria from Onyx, and Bayer HealthCare.

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