

# Tissue based biomarkers in non-clear cell RCC: Correlative analysis from the ASPEN clinical trial

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**B**iomarkers are needed in patients with non-clear cell renal cell carcinomas (NC-RCC), particularly papillary renal cell carcinoma, in order to inform on initial treatment selection and identify potentially novel targets for therapy. We enrolled 108 patients in ASPEN, an international randomized open-label phase 2 trial of patients with metastatic papillary, chromophobe, or unclassified NC-RCC treated with the mTOR inhibitor everolimus (n=57) or the vascular endothelial growth factor (VEGF) receptor inhibitor sunitinib (n=51), stratified by MSKCC risk and histology. The primary endpoint was overall survival (OS) and secondary efficacy endpoints for this exploratory biomarker analysis were radiographic progression-free survival (rPFS) defined by intention-to-treat using the RECIST 1.1 criteria and radiographic response rates. Tissue biomarkers (n=78) of mTOR pathway activation (phospho-S6 and -Akt, c-kit) and VEGF pathway activation (HIF-1 $\alpha$ , c-MET) were prospectively explored in tumor tissue by immunohistochemistry prior to treatment and associated with clinical outcomes. We found that S6 activation was more common in poor-risk NC-RCC tumors and S6/Akt activation was associated with worse PFS and OS outcomes with both everolimus and sunitinib, while c-kit was commonly expressed in chromophobe tumors and associated with improved outcomes with both agents. C-MET was commonly expressed in papillary tumors and was associated with lower rates of radiographic response but did not predict PFS for either agent. In multivariable analysis, both pAkt and c-kit were statistically significant prognostic biomarkers of OS. No predictive biomarkers of treatment response were identified for clinical outcomes. Most biomarker subgroups had improved outcomes with sunitinib as compared to everolimus.

are type 2 papillary and unclassified NC-RCC given their more aggressive disease course<sup>1</sup>.

We and others have recently reported on randomized prospective clinical trials comparing the vascular endothelial growth factor (VEGF) sunitinib with the mTOR inhibitor everolimus in patients with metastatic NC-RCC (ASPEN and ESPN)<sup>3,4</sup>. In these trials, sunitinib provided superior response rates and more durable control of disease; however, outcomes were heterogeneous based on histologic subtypes. For example, patients with papillary RCC and unclassified RCC, as well as those patients with good/intermediate risk disease had superior outcomes with sunitinib, while patients with chromophobe RCC and those with poor risk disease had superior outcomes with everolimus<sup>3</sup>. We recently reported differential outcomes based on differential plasma angiokine and immunokine levels in this setting, which were quite heterogeneously expressed according to disease risk and histology and over time during treatment resistance<sup>5</sup>. These data support the concept that these non-clear cell tumors should be regarded as distinct molecular and phenotypic entities with distinct treatment outcomes with molecularly targeted therapies, and has

## INTRODUCTION

Non-clear cell renal cell carcinoma (NC-RCC) comprises a genetically and histologically diverse set of cancers, including type 1 and 2 papillary renal cell carcinoma (RCC), chromophobe

RCC, translocation carcinoma, as well as many other rare subtypes, some of which remain histologically unclassified<sup>1,2</sup>. NC-RCC accounts for about 25% of all cases of RCC. However, in the metastatic setting, the subtypes of NC-RCC that are most commonly found

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N (%)	Baseline Data (n=78)	No Baseline Data (n=30)	Total (N=108)
<b>Treatment</b>			
Sunitinib	36 (46.2)	15 (50.0)	51 (47.2)
Everolimus	42 (53.8)	15 (50.0)	57 (52.8)
<b>Median Age (years, min - max)</b>	63 [23 - 89]	59 [27 - 100]	63 [23 - 100]
<b>Male Gender</b>	53 (67.9)	28 (93.3)	81 (75.0)
<b>Ethnicity</b>			
Hispanic/Latino	2 (2.6)	0 (0)	2 (1.9)
Not Hispanic/Latino	75 (96.2)	29 (96.7)	104 (96.3)
Missing	1 (1.3)	1 (3.3)	2 (1.9)
<b>Race</b>			
White	69 (88.5)	25 (83.3)	94 (87.0)
Black	9 (11.5)	3 (10.0)	12 (11.1)
Other	0 (0.0)	2 (6.7)	2 (1.9)
<b>Histologic Subtype</b>			
Papillary Type I	3 (3.8)	3 (10.0)	6 (5.6)
Papillary Type II	21 (26.9)	5 (16.7)	26 (24.1)
Papillary Unspecified	18 (23.1)	11 (36.7)	29 (26.9)
Chromophobe	13 (16.7)	3 (10.0)	16 (14.8)
Poorly Differentiated	6 (7.7)	2 (6.7)	8 (7.4)
Mixed Papillary/Chromophobe	1 (1.3)	0 (0.0)	1 (0.9)
Other	16 (20.5)	6 (20.0)	22 (20.4)
<b>Motzer Risk Score</b>			
Good	23 (29.5)	6 (20.0)	29 (26.9)
Intermediate	44 (56.4)	20 (66.7)	64 (59.3)
Poor	11 (14.1)	4 (13.3)	15 (13.9)
<b>Median Overall Survival (months, 95% CI)</b>	22.2 (14.8 - 39.5)	13.1 (9.3 - NR)	18.2 (13.2 - 36.2)
<b>Median Progression-free Survival (months, 95% CI)</b>	6.0 (5.6 - 8.4)	6.4 (4.3 - 11.1)	6.0 (5.6 - 8.2)
<b>Objective Response</b>	11 (14.1)	3 (10.0)	14 (13.0)

**Table 1 |** Baseline characteristics of patients included in the present correlative IHC study as compared to those patients without available biomarker data. NR indicates the estimate was not reached.

been supported by retrospective studies suggesting a subset of patients with mTOR inhibitor sensitivity<sup>6</sup>.

The identification of biomarkers predictive of treatment benefit is a major unmet need in the field of RCC therapy. In clear cell RCC, differential outcomes with immune checkpoint blockade have been observed in patients with tumors with sarcomatoid differentiation, those harboring particular immune subsets of T cell effector function, and perhaps certain complex genomic signatures<sup>7,8</sup>; however, these have not been established in non-clear cell RCC and are not commonly utilized to inform treatment selection. While histology (clear cell disease) is predictive of the benefits of high dose IL-2, and serum LDH may be predictive of the benefits of mTOR inhibition in poor risk RCC, there are no other clear predictors of treatment response or survival to specific therapies. An analysis of the RECORD-3 trial comparing sunitinib and everolimus identified several

composite prognostic circulating biomarkers for progression-free survival with everolimus, but were unable to predict overall survival and the analyses were largely restricted to clear cell RCC<sup>9</sup>. In addition, a subset of papillary RCC patients have disease that is driven by activation of the c-MET oncogene, and may benefit from c-MET inhibitors<sup>10</sup>. Furthermore, recent data from the PAMMET randomized phase 2 trial suggests that dual VEGF/c-MET targeting with cabozantinib may provide a greater probability of durable disease control as compared to sunitinib in patients with advanced papillary RCC<sup>11</sup>.

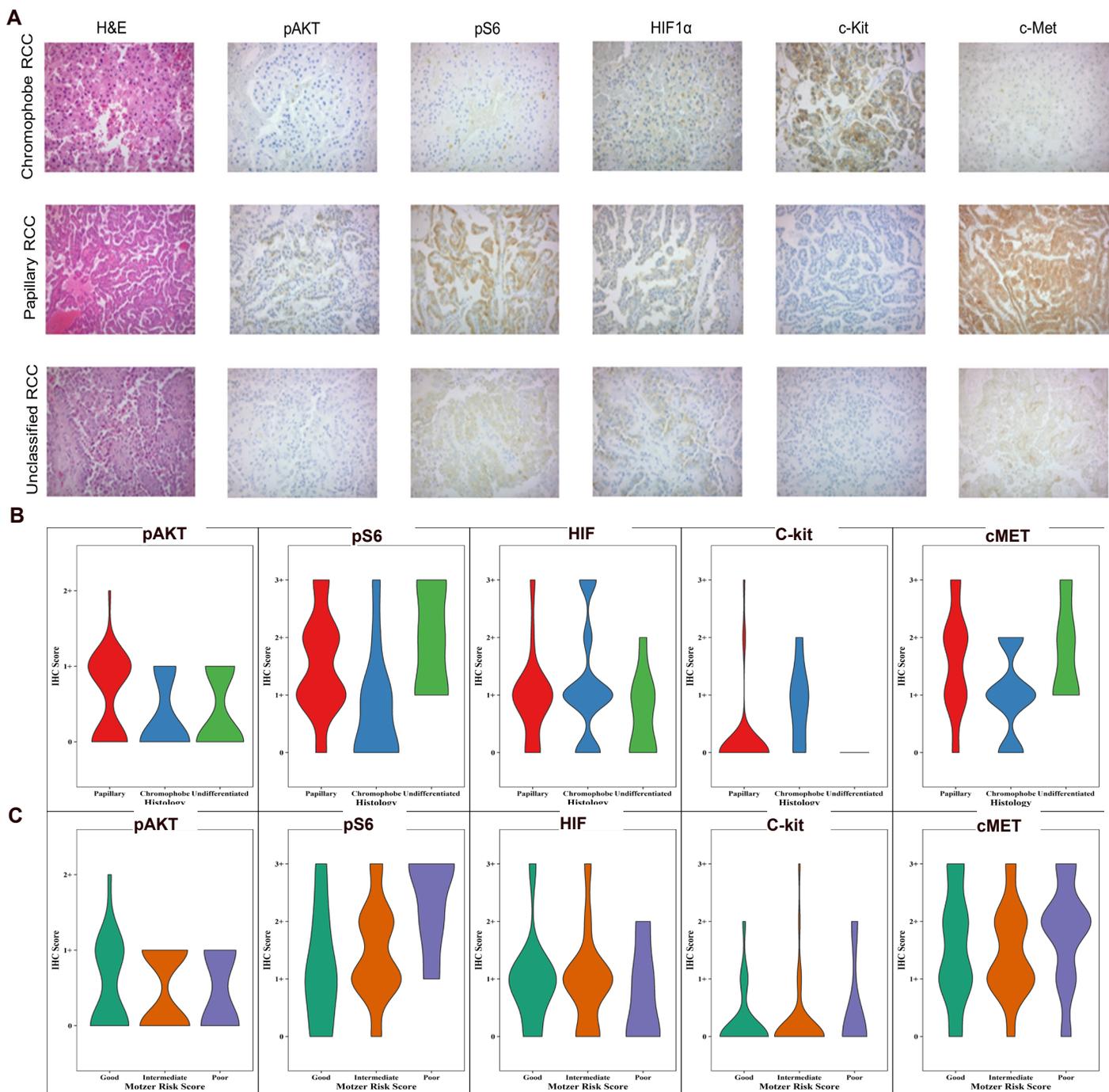
Given the heterogeneity of genomic alterations and phenotype as well as clinical outcomes of patients with metastatic non-clear cell RCC, we sought to characterize markers of specific pathway activation linked to molecularly targeted therapies. To accomplish this, we utilized tissue based protein biomarkers of mTOR

and VEGF/MET pathway activation in patients with metastatic non-clear cell RCC as part of the international, randomized, prospective clinical trial comparing sunitinib and everolimus (ASPEN). We asked whether evidence of mTOR pathway activity or VEGF-HIF-1 $\alpha$ /MET expression differed by histologic subtype and MSKCC risk group<sup>12,13</sup>, and whether clinical efficacy outcomes differed by baseline tissue pathway biomarker expression at the protein level. Based these previous studies, familial syndromes of mTOR pathway activation in chromophobe RCC<sup>14</sup> and c-met pathway activation in hereditary and sporadic papillary RCC<sup>15</sup>, and our own plasma biomarker analysis<sup>5</sup>, our specific a priori hypotheses were that pS6 and pAKT high level expression will be associated with a greater radiographic progression free survival (rPFS) by RECIST 1.1 criteria with everolimus as compared to sunitinib as well as ORR and OS; c-KIT high level expression will be associated with chromophobe histology and a greater rPFS, ORR, and OS benefit with everolimus as compared to sunitinib; and finally that HIF-1 $\alpha$  and c-MET will be associated with papillary RCC histology and will be associated with a greater rPFS, ORR, and OS benefit with sunitinib as compared to everolimus. We also suspected that high levels of pS6 and pAKT and cMET will be associated with poor outcomes overall including shorter rPFS, OS, and low ORR regardless of therapy.

We employed immunohistochemical studies of primary nephrectomy or metastatic biopsy specimens to examine the prognostic and predictive associations with progression-free and overall survival in this pre-specified prospective secondary analysis. Such findings could ideally permit the selection of patients for an mTOR or VEGF/MET treatment such as cabozantinib more optimally than histology or clinical risk score alone.

## RESULTS

From September 23, 2010 through October 28, 2013, we accrued 109 subjects across three (3) countries and 17 participating sites. One subject did not receive the study drug and

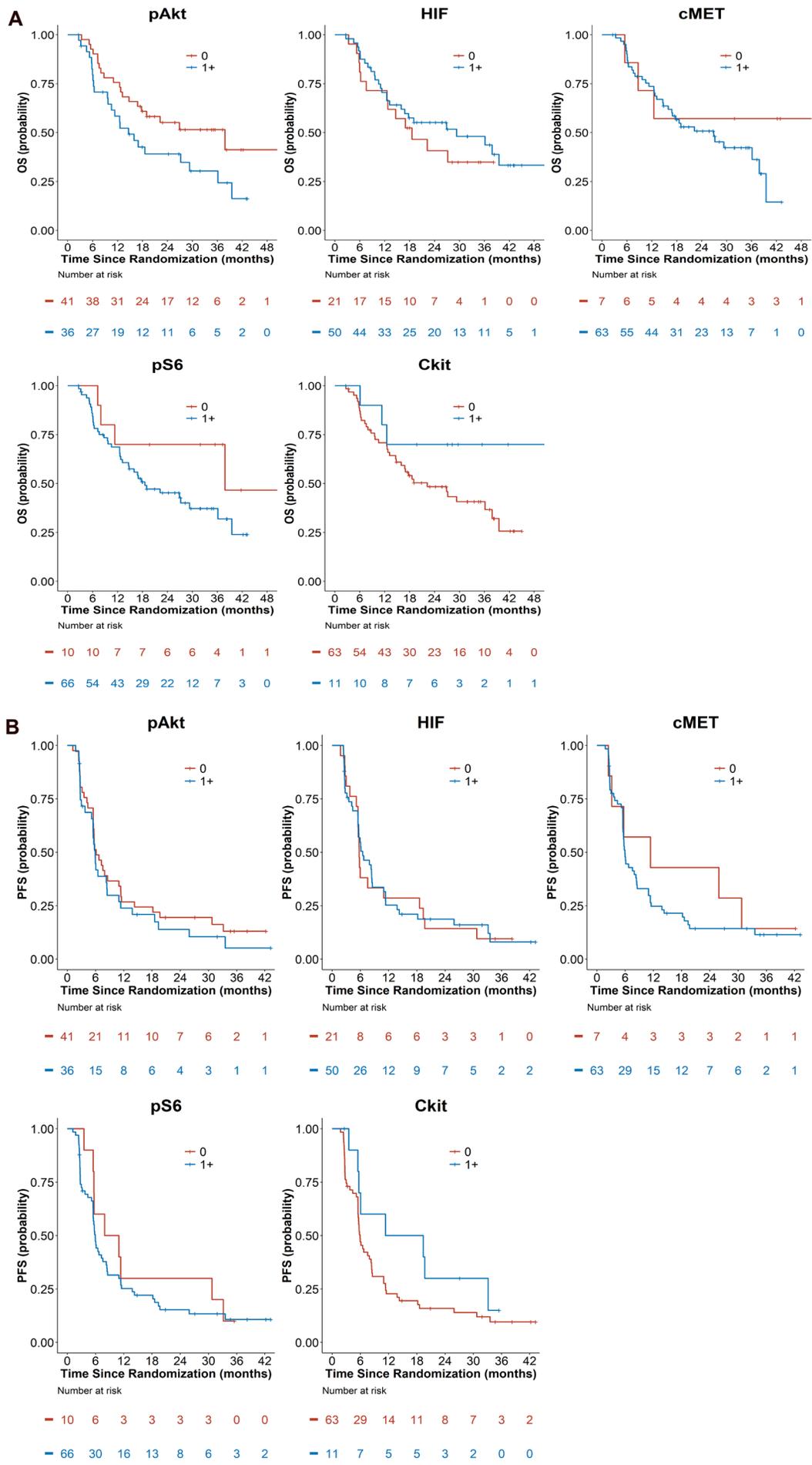


**Figure 1:** (A) Representative images of biomarker expression by immunohistochemistry from the ASPEN study according to histologic subtypes of papillary, chromophobe, and unclassified RCC. Note c-kit expression predominantly in chromophobe RCC, c-met expression in papillary RCC. (B) Distributions of tissue IHC biomarker expression levels according to histologic subtype categorized as papillary (red), chromophobe (blue), and undifferentiated (green). (C) Distributions of tissue IHC biomarker expression levels according to MSKCC risk groups coded as good (green), intermediate (orange), and poor (purple).

withdrew and was replaced, leaving 108 evaluable subjects who were then randomized to sunitinib (51 subjects) or everolimus (57 subjects). Biomarker data was available from 78 of 108 patients (72%), with over 90% of the cases derived from primary tumor tissue from nephrectomy or renal biopsy, including 36 patients treated with sunitinib and 38 patients treated with everolimus. Thirty patients (15 in each treatment group) had insufficient

tissue available for IHC studies, and are excluded from this analysis (see CONSORT diagram, [Supplementary Figure 1](#)). The data lock for the final overall survival analysis was May 2016. Patients in the biomarker evaluable population did not differ from those without evaluable biomarkers with the exception of more women (32% vs. 7%), more type 2 papillary (27% vs. 17%), and fewer intermediate MSKCC risk patients (56% vs. 67%) in the biomarker

group, respectively ([Table 1](#)). The majority of evaluable patients (42/78, 54%) had metastatic papillary RCC with non-type 1 histology; only 3 patients had type 1 papillary RCC. The second most common histologic subtype was metastatic chromophobe RCC, which accounted for 17% of patients, followed by unclassified/poorly differentiated RCC, comprising 8% of patients.



**Figure 2: (A)** Kaplan-Meier Overall Survival Curves by Tissue Biomarkers (pAkt, pS6, HIF-1 $\alpha$ , c-kit, and c-MET). **(B)** Kaplan-Meier Progression-Free Survival Curves by Tissue Biomarkers (pAkt, pS6, HIF-1 $\alpha$ , c-kit, and c-MET).

## Distribution of Tissue Protein Biomarkers

Lower protein expression scores were more common across all patients for p-Akt, HIF-1 $\alpha$ , and c-kit with 1.3%, 12.8%, and 6.4% harboring at least 2+ expression by IHC. The distribution of IHC scores was fairly balanced for p-S6 and c-MET, with 44.8% and 43.6% of patients harboring at least 2+ expression by IHC (Supplementary Table 3). Representative IHC images of each biomarker across the 3 histologic subtypes are shown in Figure 1A.

## Association of Tissue Protein Biomarkers with Histology and MSKCC Risk Group

Chromophobe patients had a greater percentage of 0 IHC values for p-Akt, p-S6, and c-MET, and as expected were more likely to have detectable (1+ or higher) c-kit expression than non-chromophobe RCC patients (62% vs. 5%) (Figure 1B, Supplementary Table 3). The distributions were fairly uniform within each group for p-S6 and c-MET. In papillary RCC, c-MET expression was absent in 9.3% of patients as compared to 23% of chromophobe and 0% of unclassified tumors. However, any expression and intense 3+ expression of c-MET was detected in 82%, 69%, and 86% in papillary, chromophobe, and unclassified tumors, respectively, while intense 3+ c-MET expression was detectable in 14%, 0%, and 18%, respectively, indicating the c-MET expression was not restricted to papillary subtypes.

Phospho-Akt, HIF-1 $\alpha$ , and c-MET scoring distributions were similar across the three MSKCC risk groups. Patients with good MSKCC risk were more likely to have absent p-S6 (26%) as compared to patients with poor MSKCC risk (0%), and less likely to have intense p-S6 staining of at least 3+ (13% vs 55%). Patients with good MSKCC risk had lower scores

Biomarker	Median OS (months, 95% CI)		N	Univariate		Multivariable	
	0	1+		HR (95% CI)	P Value	HR* (95% CI)	FDR
	pAkt	37.9 (17.7-NR)		14.7 (10.5-36.2)	77	1.8 (1.0-3.3)	0.055
pS6	37.9 (11.3-NR)	18.6 (13.2-39.5)	76	2.1 (0.8-6.0)	0.149	1.5 (0.4-5.0)	0.529
HIF-1α	18.6 (12.6-NR)	29.3 (16.0-NR)	71	0.8 (0.4-1.5)	0.410	0.8 (0.4-1.6)	0.529
c-kit	22.2 (14.8-39.5)	NR (12.5-NR)	74	0.4 (0.1-1.3)	0.131	0.1 (0.0-0.7)	0.056
c-MET	NR (8.7-NR)	26.9 (16.8-NR)	70	1.9 (0.6-6.5)	0.302	1.9 (0.5-7.2)	0.529

Biomarker	Median PFS (months, 95% CI)		N	Univariate		Multivariable	
	0	1+		HR (95% CI)	P Value	HR* (95% CI)	FDR
	pAkt	6.1 (5.6-11.3)		5.9 (5.5-11.0)	77	1.2 (0.7-2.0)	0.431
pS6	9.5 (5.7-NR)	5.9 (5.5-8.4)	76	1.3 (0.7-2.7)	0.438	1.2 (0.5-2.9)	0.877
HIF-1α	5.7 (5.5-19.4)	6.5 (5.6-11.0)	71	1.0 (0.6-1.7)	0.927	1.1 (0.6-2.0)	0.877
c-kit	5.9 (5.5-8.4)	15.4 (5.7-NR)	74	0.6 (0.3-1.2)	0.155	0.4 (0.2-1.1)	0.333
c-MET	11.4 (3.2-NR)	5.9 (5.5-8.5)	70	1.4 (0.6-3.3)	0.447	1.1 (0.4-2.7)	0.899

**Table 2: (A)** Median overall survival (OS) by tissue biomarkers for all evaluable patients. Univariate and multivariable hazard ratios of OS for each biomarker. Cut-point of 1+ scoring. NR indicates the estimate was not reached. \*Adjusting for treatment arm and stratification variables (histology and MSKCC risk groups) **(B)** Median progression-free survival (PFS) by tissue biomarkers for all evaluable patients. Univariate and multivariable hazard ratios of PFS for each biomarker. Cut-point of 1+ scoring. NR indicates the estimate was not reached.

of p-S6 while relatively more poor risk patients had higher IHC scores for this biomarker (Figure 1, Supplementary Table 3). Thus, neither c-MET nor p-AKT staining distinguished risk groups, while downstream p-S6 was clearly associated with poor risk disease.

### Associations of Tissue Biomarkers with Clinical Outcomes

There were 67 PFS events and 44 deaths in 78 patients with evaluable tissue biomarker data and as of the final data lock in May 2016, the median follow-up time in 34 alive patients was 29 months (range=2.6-55.7). Patients with 1+ pAkt tumor tissue staining had a shorter median OS (14.7 months) as compared with patients with absent p-Akt (37.9 months). However, none of the five tissue biomarkers were prognostic of OS in univariate analysis (Figure 2A and Table 2).

In multivariable analysis of OS, however, both p-Akt and c-kit were statistically significant prognostic biomarkers of OS after multiplicity adjustment and adjustment for histologic type and MSKCC risk. The

multivariable hazard ratio (HR) for death for p-Akt was 2.2 (95% CI=1.1-4.2, FDR=0.056). On the other hand, detection of c-kit was associated with improved survival (HR=0.1;95% CI=0.0-0.7; FDR=0.056) irrespective of histology.

None of the tissue biomarkers were associated with PFS overall (Table 2B, Figure 2B). Additionally, when exploring a higher threshold cut-off for IHC positivity of 2-3+ expression, none of the biomarkers had statistically significant associations with OS or PFS in secondary analyses comparing biomarker expression 0-1 versus >2+ (Supplementary Tables 5A and 5B).

### Predictive Associations with Clinical Outcomes

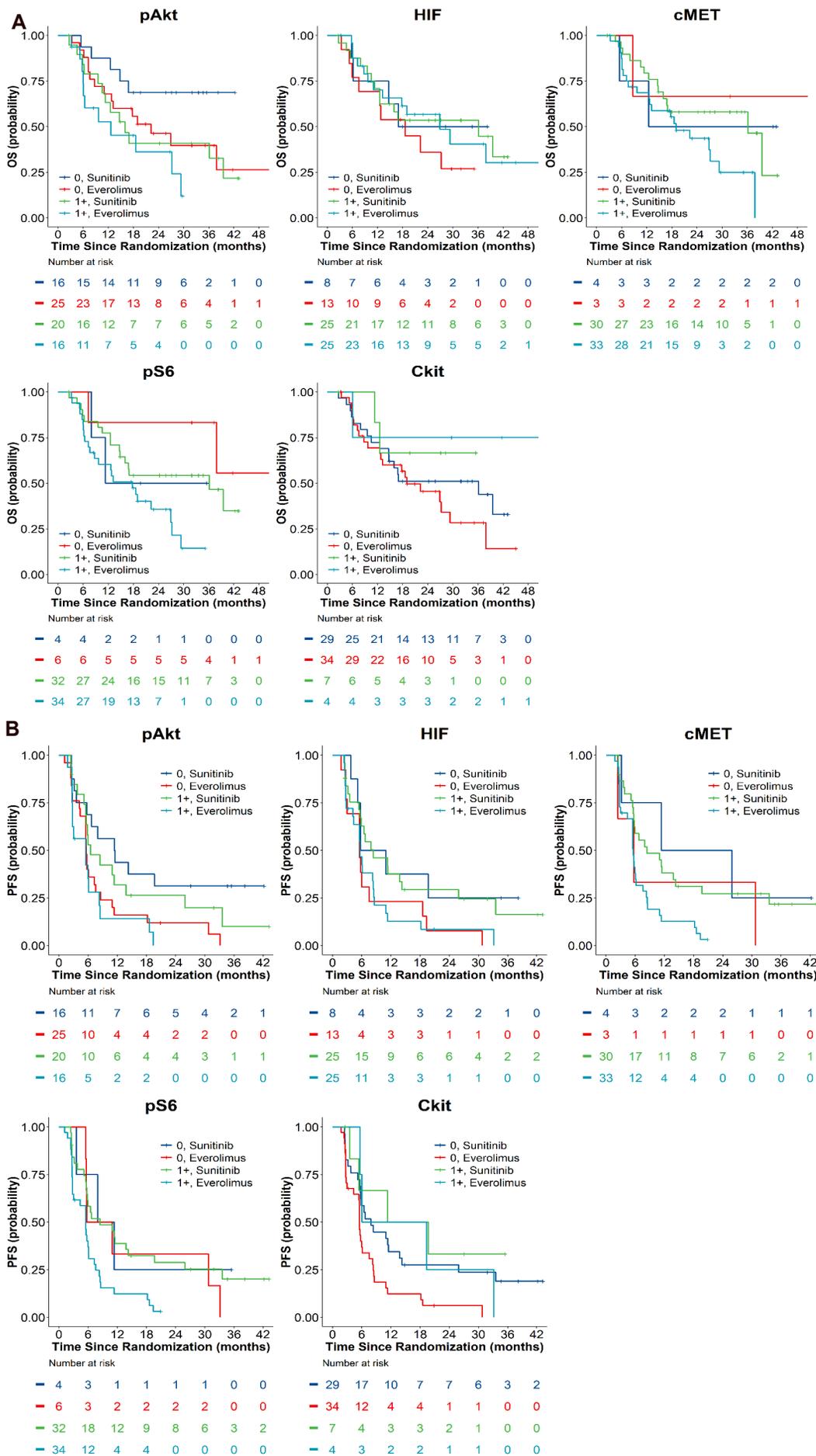
Finally, we examined each of the 5 pathway-based protein biomarkers for associations with outcomes of either sunitinib or everolimus and the predictive value of biomarker expression for superiority of one therapy over the other. None of the tissue biomarkers were predictive of treatment benefit for OS or PFS for sunitinib or everolimus (Tables 3A and 3B) regardless of the

IHC scoring thresholds (Supplementary Tables 5A and 5B). Lastly, while none of the biomarkers were predictive of differential objective response (Tables 4B and Supplementary 7B), we did note that patients with c-MET expressing tumors had a lower objective response rate by RECIST 1.1 (11% ORR) as compared to patients with tumors lacking c-MET expression (43% ORR). In sunitinib treated patients, the ORR was 17% vs 50% in patients with c-MET expressing vs. non-expressing tumors, while in everolimus treated patients, the ORR was 6% vs. 33% respectively. The ORR for patients with high c-kit expression was 0% for sunitinib vs. 24% for patients with absent c-kit expression, as compared to the opposite result for everolimus, which

had an ORR of 25% for patients with high c-kit expression as compared to 6% for patients that lacked c-kit expression.

### DISCUSSION

The treatment of patients with metastatic non-clear cell RCC continues to evolve and improve. Based on the ASPEN and ESPN randomized trials, sunitinib was demonstrated to have more prolonged progression free and overall survival and higher objective response rates as compared to everolimus<sup>3,4</sup> overall and particularly in favorable/intermediate risk and papillary/unclassified subtypes. However, everolimus had clear activity and improved outcomes in patients with poor risk disease and chromophobe RCC variants, mirroring prior prospective data derived from the global phase 3 temsirolimus trial. Recently cabozantinb was shown to have superior responses and PFS as compared to sunitinib in advanced papillary RCC (both type 1 and 2), suggesting that dual inhibition of c-MET and VEGF may provide more durable clinical benefits<sup>11</sup>. Here we sought to identify subgroups of patients



**Figure 3: (A)** Kaplan-Meier Overall Survival Curves by Treatment Assignment and Tissue Biomarkers (pAkt, pS6, HIF-1 $\alpha$ , c-kit, and c-MET). **(B)** Kaplan-Meier Progression-Free Survival Curves by Treatment Assignment and Tissue Biomarkers (pAkt, pS6, HIF-1 $\alpha$ , c-kit, and c-MET).

that may differentially respond to molecularly targeted therapies through the use of protein-based assays of potential driver pathways. While we found that activation of the mTOR pathway including low level Akt and S6 phosphorylation was associated with poor risk disease and worse survival, these biomarkers were not sufficiently predictive of clinical benefit for everolimus compared to sunitinib. While chromophobe patients with high c-kit expression had a numerically higher ORR with everolimus than sunitinib, this did not translate into longer PFS or OS potentially due to the relatively small sample size of this subgroup.

Recently, we identified specific subsets of non-clear cell RCC patients that have poor outcomes in the ASPEN trial based on levels of plasma angiokines associated with angiogenesis, metastasis, and immune evasion, particularly osteopontin (OPN), TIMP-1, thrombospondin-2, hepatocyte growth factor (HGF), and VCAM-116. These data suggest potential therapeutic targets associated with disease burden and treatment resistance. We could not directly assess most of these biologic features in tumor tissues, and thus cannot correlate tumor angiokine expression with plasma levels and clinical outcomes. However, when we could there was not a clear correlation with outcomes. For instance, we evaluated c-met, the receptor for hepatocyte growth factor, in tumors and found no correlation with c-met levels and clinical outcomes in non-clear cell as well as the subset of papillary RCC patients treated with sunitinib or everolimus, despite a prognostic association of high plasma HGF levels with poor overall survival<sup>5</sup>. Other assessments of pathway addiction such as c-met phosphorylation or amplification or splice variants, or mTOR pathway mutations<sup>17,18</sup>, should be further evaluated against specific targeted therapy outcomes in this

**A**

Biomarker	Median OS (months, 95% CI)				N	Univariate P Value	Multivariable FDR
	Sunitinib		Everolimus				
	0	1+	0	1+			
pAkt	NR (16.8-NR)	16.0 (11.3-NR)	22.2 (12.7-NR)	12.6 (6.1-NR)	77	0.560	0.717
pS6	11.3 (8.0-NR)	36.2 (14.8-NR)	NR (37.9-NR)	17.7 (8.7-29.3)	76	0.101	0.717
HIF-1α	17.0 (14.7-NR)	36.2 (12.5-NR)	18.6 (7.6-NR)	26.9 (13.2-NR)	71	0.530	0.717
c-kit	36.2 (14.8-NR)	NR (12.5-NR)	19.0 (12.7-NR)	NR (6.1-NR)	74	0.473	0.717
c-MET	12.5 (5.5-NR)	36.2 (16.8-NR)	NR (8.7-NR)	19.0 (12.7-NR)	70	0.429	0.717

**B**

Biomarker	Median PFS (months, 95% CI)				N	Univariate P Value	Multivariable FDR
	Sunitinib		Everolimus				
	0	1+	0	1+			
pAkt	11.4 (5.7-NR)	6.5 (5.6-NR)	5.7 (5.5-8.6)	5.5 (2.8-18.6)	77	0.662	0.868
pS6	9.6 (3.5-NR)	8.4 (5.7-25.9)	8.3 (5.7-NR)	5.5 (3.0-7.3)	76	0.489	0.778
HIF-1α	8.4 (5.7-NR)	8.4 (5.9-33.6)	5.6 (3.0-NR)	5.7 (4.4-8.6)	71	0.735	0.869
c-kit	8.0 (5.8-14.3)	15.5 (5.5-NR)	5.5 (5.5-8.3)	12.7 (5.7-NR)	74	0.698	0.778
c-MET	18.6 (3.2-NR)	8.4 (5.8-19.7)	5.7 (2.4-NR)	5.6 (5.5-7.6)	70	0.957	0.778

**Table 3: (A)** Median overall survival (OS) by treatment group and tissue IHC biomarkers using a cut-point of 1+, including biomarker-treatment interaction p-values. NR indicates the estimate was not reached. **(B)** Median progression-free survival (PFS) by treatment group and tissue IHC biomarkers using a cut-point of 1+, including biomarker-treatment interaction p-values. NR indicates the estimate was not reached.

context.

Our analysis has several limitations. The first is the heterogeneous nature of our patient population, comprised of multiple tumor types with likely widely differing genotypes and biomarker expression profiles and differing clinical risk groups. This limits our power to determine predictive interactions for individual subgroups and therapies. The second is the current lack of genotyping data in this trial at the present time, which does not permit a more detailed molecular analysis of pathway mutations, amplifications, splice variants, or expression. We chose to focus our biomarker studies for the present analysis on protein and phosphoproteomic alterations given that the functional consequences of the known genomic alterations is frequently unknown, and we hypothesized that these pathway-based protein assays would be more functionally relevant to drug activity for therapies targeting the mTOR or VEGF pathways. Third is the lack of present information in this trial on the activity of other pathways, such as the NRF2/KEAP1, fumarate hydratase and other metabolic regulators, or epigenetic regulators. Further investigation into these and other key biologic processes including the immune landscape of these tumors

may shed light into future therapeutic directions, including combination VEGF/c-MET and immune checkpoint blockade or novel approaches.

Our work has several strengths, including being the largest, prospective global trial conducted to date in this metastatic non-clear cell RCC population. We mandated tissue collection as part of eligibility, which ensured a robust program for biomarker study, and we utilized previously validated IHC assays with appropriate validated controls. Our pathologists were blinded to outcome, while our statisticians performed the clinical analysis while blinded to biomarker studies, ensuring a lack of bias in the data analysis plan. While the trial was open label for treatment, treatment was randomized and not selected based on any patient or tumor characteristics. IHC studies are relatively easy to conduct in clinical practice relative to complex genotyping assays, and thus this work could be readily applicable if successful. Finally, we conducted long term follow up to ensure an adequate number of events for the gold standard of overall survival as an endpoint.

In conclusion, we demonstrate the negative prognostic value for Akt pathway activation in non-clear cell RCC and the positive prognostic value for c-kit expression in a prospective

clinical trial of sunitinib vs. everolimus. Additionally, we show that c-MET expression is associated with a poor response to sunitinib or everolimus, while c-kit expression is associated with a better response to everolimus. However, we were unable to show a predictive treatment-biomarker interaction using the 5 pathway-directed biomarkers in this study, and thus, overall sunitinib remained the superior therapy in the ASPEN trial.

## MATERIALS & METHODS

### Study design and patients

This was a prospective, open-label randomized United States Food and Drug Administration IND-exempt trial conducted across 17 participating global sites, including the United States, Canada, and the United Kingdom. Regulatory oversight in Canada and the UK was obtained for this trial. After meeting eligibility, randomized subjects were assigned 1:1 to either sunitinib malate or everolimus at approved doses until disease progression.

Patients were eligible if they had histologically confirmed advanced RCC with non-clear cell pathology after local site review by pathology, including unclassified subtypes. Mixtures of these non-clear cell variants were allowed provided they consisted predominantly (> 50%) of papillary, chromophobe or undifferentiated histology. Patients with minor clear cell components (<50%) were permitted provided the dominant histology and presumed primary histology was non-clear cell. Exclusion criteria for the study included active untreated CNS metastases, prior systemic therapy for RCC, and collecting duct or medullary histology. Full eligibility details are provided in the primary clinical manuscript<sup>3</sup>.

This study was registered as an International Standard Randomised Controlled Trial with ClinicalTrials.gov number NCT01108445. All patients provided informed consent under an

**A**

Biomarker	0 N (%)	1+ N (%)	N	OR (95% CI)
pAkt	6 (14.6)	5 (13.9)	77	0.9 (0.2 – 3.4)
pS6	1 (10.0)	10 (15.2)	76	1.6 (0.3 – 31.3)
HIF-1 $\alpha$	3 (14.3)	7 (14.0)	71	1.0 (0.2 – 4.9)
c-kit	9 (14.3)	1 (9.1)	74	0.6 (0.0 – 3.8)
c-MET	3 (42.9)	7 (11.1)	70	0.2 (0.0 – 1.0)

**B**

Biomarker	Sunitinib			Everolimus		
	N	0 N (%)	1+ N (%)	N	0 N (%)	1+ N (%)
pAkt	36	3 (18.8)	4 (20.0)	41	3 (12.0)	1 (6.2)
pS6	36	0 (0.0)	7 (21.9)	40	1 (16.7)	3 (8.8)
HIF-1 $\alpha$	33	1 (12.5)	6 (24.0)	38	2 (15.4)	1 (4.0)
c-kit	36	7 (24.1)	0 (0.0)	38	2 (5.9)	1 (25.0)
c-MET	34	2 (50.0)	5 (16.7)	36	1 (33.3)	2 (6.1)

**Table 4:** (A) Objective response rate showing N (%) and odds ratio (95% CI) by IHC 0 vs. 1+ biomarker status. (B) Objective response rate by treatment assignment and IHC 0 vs. 1+ biomarker status.

institutional IRB-approved consent form. This was an investigator-initiated study, with the Duke Cancer Institute as lead coordinating center and biorepository. A contract research organization, inVentiv Health Clinical, oversaw the collection of data and safety monitoring on behalf of Duke globally.

### Tissue Biomarker Studies

Primary nephrectomy or metastatic biopsy specimens were prospectively collected on all patients as part of the eligibility criteria for the ASPEN trial. Formalin-fixed paraffin embedded tissue was collected and underwent IHC studies for 5 biomarkers: phospho-S6 and phospho-Akt as measures of mTOR pathway activation; c-kit as a defining biomarker of chromophobe RCC which has been associated with mTOR pathway activation through folliculin mutations 14; c-MET total expression; and HIF-1 $\alpha$  as a measure of VEGF pathway activation. The specific antibodies utilized and validated on control tissues, their concentration/dilution, and methods used are described in [Supplementary Table 1](#). Investigators and statisticians were blinded to the results of these biomarker studies at the time of outcome analysis.

### Outcomes

The primary endpoint of this tissue biomarker study was overall survival (OS), defined as the interval from date of random assignment until date of death or date of last follow-up. A key secondary outcome included radiographic progression-free survival (PFS), defined as the time from date of random assignment until date of disease progression (by RECIST 1.1 criteria), a new primary malignancy, or death, whichever occurred first. Other pre-specified efficacy secondary endpoints included radiographic response rates per RECIST 1.1, and clinical benefit response (CBR), defined as the composite sum of partial response, complete response, and prolonged stable disease for more than 6 months. Objective response rate (ORR) was defined as the sum of complete and partial response by RECIST 1.1.

### Statistical analysis

The five tissue biomarkers (phospho-S6, phospho-Akt, c-kit, HIF-1 $\alpha$ , and c-MET) include the IHC scores of 0, 1+, 2+, or 3+. Missing data from the 78 evaluable patients were excluded from the analyses and resulted from either an insufficient amount of tumor to categorize the sample, the sample being of an unacceptable quality, or a lack of

tissue provided by the patient. All five tissue biomarkers were dichotomized and analyzed using two pre-specified cut-points in the statistical analysis plan. The primary analysis was based on 0 vs. 1+ whereas the secondary analysis was 0-1 vs. 2+, where the “1+” group included scores of 1+, 2+, and 3+, and the “2+” group included scores of 2+ and 3+. The proportional hazards model was utilized to determine the prognostic importance of the tissue biomarkers in predicting OS and PFS adjusting for the treatment arm and the stratification factors (histologic type and MSKCC risk groups). The association of each biomarker with OS and PFS was summarized with a hazard ratio (HR) and 95% confidence interval (CI) for this exploratory analysis, while p-values were adjusted for multiplicity using the false discovery rate (FDR) of 0.056, and we considered FDR < 0.1 to be statistically significant. Additionally, the proportional hazards model was used to test for each of the tissue biomarker-treatment interaction terms in predicting OS and PFS. The Kaplan-Meier approach was used to estimate the OS and PFS distributions.

When assessing the association of the biomarkers with histologic subtype, we classified all papillary tumors, including types I and II, as “papillary.” Chromophobe tumors were designated “chromophobe,” and the remaining 30 patients fell into the “undifferentiated” category. Patients with an MSKCC risk score of 0 were classified as having “good” risk, while patients who had MSKCC risk scores of either 1 or 2 were assigned to the “intermediate” group, and those with a score of 3 or above were categorized as “poor.”

Furthermore, logistic regression analysis was used to test for the prognostic importance of the tissue biomarkers in predicting objective response rate. Odds ratios (OR) and 95% confidence intervals (CI) summarized these findings. The final statistical analysis plan was approved by the Duke IRB on August 14, 2014. All analyses were performed using R version 3.5.3 and were adjusted for multiplicity using the false discovery rate (FDR) in determining whether any of tissue biomarkers were prognostic or predictive of OS or PFS.

### SUPPLEMENTAL INFORMATION

Any supplementary information

including supplementary figures and supplementary tables, legends, respective materials and methods, conflicts of interest, other relevant data can be found online at:

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## AUTHOR CONTRIBUTIONS

AJA: literature search, figures, study design, data analysis and interpretation, writing;  
SH: data analysis and interpretation, study design, figures, writing;  
QY, SZ, AC: data analysis and interpretation, figures, writing  
TE, WMS, RJJ, JAG, UNV, JP, REH, JDH, CKK, TFL, IP, LMP, CWR, AP: data collection, analysis, and interpretation, writing;  
WCF: data collection and analysis, writing;  
DJG: literature search, figures, study design, data analysis and interpretation, writing.

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