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Clear Cell Renal Cell Carcinoma Subtypes Identified by BAP1 and PBRM1 Expression

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Abstract

Purpose—In clear cell renal cell carcinoma *BAP1* and *PBRM1* are 2 of the most commonly mutated genes (10% to 15% and 40% to 50%, respectively). We sought to determine the prognostic significance of PBRM1 and BAP1 expression in clear cell renal cell carcinoma.

Materials and Methods—We used immunohistochemistry to assess PBRM1 protein expression in 1,479 primary clear cell renal cell carcinoma tumors that were previously stained for BAP1. A centralized pathologist reviewed all cases and categorized tumors as positive or deficient for PBRM1 and BAP1. Kaplan-Meier and Cox regression models were used to evaluate association of PBRM1 and BAP1 expression with the risk of death from renal cell carcinoma and the risk of metastasis after adjustment for age and the Mayo Clinic SSIGN (stage, size, grade and necrosis) score.

Results—PBRM1 and BAP1 expression was PBRM1+ BAP1+ in 40.1% of tumors, PBRM1– BAP1+ in 48.6%, PBRM1+ BAP1– in 8.7% and PBRM1– BAP1– in 1.8%. The incidence of PBRM1 and BAP1 loss in the same tumor was significantly lower than expected (actual 1.8% vs expected 5.3%, $p < 0.0001$). Compared to patients with PBRM1+ BAP1+ tumors those with PBRM1– BAP1+ lesions were more likely to die of renal cell carcinoma (HR 1.39, $p = 0.035$), followed by those with PBRM1+ BAP1– and PBRM1– BAP1– tumors (HR 3.25 and 5.2,

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respectively, each $p < 0.001$). PBRM1 and BAP1 expression did not add independent prognostic information to the SSIGN score.

Conclusions—PBRM1 and BAP1 expression identified 4 clinical subgroups of patients with clear cell renal cell carcinoma who had divergent clinical outcomes. The clinical value of these biomarkers will be fully realized when therapies targeting pathways downstream of PBRM1 and BAP1 are developed.

Keywords

kidney; carcinoma; renal cell; genes; tumor suppressor; biological markers; mortality

Patients with ccRCC show widely divergent clinical behavior, which is likely explained by genetic differences in the tumor. Genes implicated in ccRCC pathogenesis at a frequency of between 3% and 50% include *PBRM1*, *BAP1*, *SETD2*, *TCEB1* and *KDM5C*.^{1–10} The impact of these mutations on ccRCC clinical outcomes remains unclear.

Two of the most commonly mutated genes in ccRCC are *PBRM1* (about 40% to 50%) and *BAP1* (10% to 15%). Both genes are located on chromosome 3p, which is the most commonly (about 90%) cytogenetically deleted region in ccRCC. Previous studies intimate that mutations in *PBRM1* and *BAP1* are largely mutually exclusive, suggesting a possible genetic interaction between these genes.^{8,10} Previously we found that patients whose tumor harbors a *PBRM1* mutation had improved outcomes compared to those whose tumor harbors a *BAP1* mutation.¹⁰ Separate investigators, including those using data from TCGA (The Cancer Genome Atlas), confirmed that mutations in *BAP1* are associated with a poor prognosis but mutations in *PBRM1* did not impact prognosis compared to wild-type *PBRM1*.⁶

Complementing the analysis of the clinical significance of mutations in *PBRM1* and *BAP1* in ccRCC, multiple groups, including ours, assessed the association of PBRM1 and BAP1 protein expression with clinical outcomes. We previously developed and validated IHC based assays with a high degree of reliability for detecting mutations in these genes (ie if the stain is negative, the gene is mutated).⁸ Using IHC we noted that BAP1 loss was associated with a significant increase in the risk of RCC specific death.¹¹ In addition at least 2 studies demonstrate that loss of PBRM1 expression on IHC is associated with worse outcomes.^{12,13}

Previous studies of the clinical significance of *PBRM1* and *BAP1* mutations in ccRCC are limited. 1) Relatively small sample sizes with short-term followup made it difficult to detect small differences in clinical outcomes or differences that might develop later. 2) Many prior groups analyzed the clinical significance of PBRM1 or BAP1 individually rather than both proteins simultaneously in the same cohort. In the current investigation we addressed the limitations of the prior studies by assessing PBRM1 and BAP1 in a large sample of greater than 1,400 cases with extended followup, demonstrating that PBRM1 and BAP1 are interrelated and identifying 4 subtypes of ccRCC.

MATERIALS AND METHODS

Patient Selection

After receiving institutional review board approval we identified 1,479 patients in the Mayo Clinic Rochester nephrectomy registry who presented with nonmetastatic disease and were treated with radical nephrectomy or nephron sparing surgery for unilateral, sporadic, non-cystic ccRCC between 1990 and 2009. Followup data and clinicopathological covariates were abstracted from the registry. Briefly these data are routinely updated and maintained through a combination of active (mailed questionnaires) and passive (medical record and linkage to national databases) surveillance by experienced clinical coordinators. Pathological features were analyzed in standardized fashion by 1 urological pathologist (JCC) who centrally reviewed the microscopic hematoxylin and eosin slides from all specimens while blinded to patient outcome. As part of the review the pathologist determined components of the Mayo Clinic SSIGN score, an externally validated prognostic algorithm for ccRCC prognosis. A higher score implies poorer cancer specific survival.

Immunohistochemistry Assay Methodology

A representative formalin fixed, paraffin embedded tissue block with viable tumor was selected from each case. From each block serial 3 to 4 μm unstained sections were obtained and submitted for IHC staining. IHC was performed with the Benchmark XT™ automated stainer as previously described.^{8,10,11} Briefly sections were deparaffinized, rehydrated and subjected to heat induced epitope retrieval. They were incubated with primary antibody against BAP1 (mouse monoclonal, clone C-4, Santa Cruz Biotechnology®) or PBRM1 (rabbit polyclonal, Bethyl Laboratories, Montgomery, Texas). After incubation slides were subjected to a DAB detection system (Ventana Medical Systems, Tucson, Arizona), counterstained with hematoxylin, dehydrated back to xylene and coverslipped. Three positive and negative ccRCCs with known mutation status served as controls for each immunostain run. Nuclear reactivity was considered a positive signal for BAP1 and/or PBRM1. In each tumor section lymphocytes, stromal fibroblasts and endothelial cells served as internal positive control cells.

Immunohistochemistry

Assay Validation—IHC assays for BAP1 and PBRM1 were validated using 176 genetically characterized ccRCC samples.⁸ As previously reported, scoring was performed by a genitourinary pathologist (PK) blinded to genotype. Briefly of the 176 ccRCC cases BAP1 IHC could be interpreted in 175, including all 148 *BAP1* wild-type cases that demonstrated positive BAP1 nuclear staining as well as 2 that were *BAP1* mutant. All 22 cases that were *BAP1* mutant stained negative for BAP1 as well as 3 that were *BAP1* wild-type. The correlation between BAP1 IHC and *BAP1* mutation status was highly statistically significant ($p = 3 \times 10^{-58}$). With respect to PBRM1 87% of samples that were IHC negative had *PBRM1* mutations and 91% of IHC positive samples were wild-type ($p = 4 \times 10^{-23}$).

Analysis—A centralized pathologist (PK) blinded to clinicopathological variables reviewed all IHC slides. Tumors were categorized as PBRM1 (or BAP1) positive when tumors expressed strong diffuse nuclear staining and PBRM1 (or BAP1) negative when

tumor cells showed a diffuse lack of nuclear staining. In a small subset of cases the staining pattern was not classic. In some tumors only a distinct tumor nodule/area showed absent nuclear staining and these focal negative areas were thought to represent subclones of the tumor with genetic heterogeneity. A fourth pattern was seen with tumor cells showing weak nuclear staining. This weak positive pattern could have been secondary to preanalytical variables or IHC detection of protein (functional or altered) expressed in smaller quantities. Since IHC results in the positive and negative cohorts were unequivocal, we performed our primary analysis on this cohort.

As part of secondary analysis we grouped weakly positive cases with positive cases and focal negative cases with negative cases. For tumors labeled negative, 100% of tumor cells were negative for the protein. In focal negative cases a subclone was negative and the remaining tumor cells were positive. As some tumor cells had lost the stain and, therefore, did not have the protein, they were grouped with negative cases. Weak positive cases showed less intense staining in all tumor cells. Since there was staining and, therefore, expression of some protein, these cases were grouped with positive cases.

Statistical Analysis

Clinical and pathological data were compared among patients with PBRM1 and BAP1 positive and negative tumors using the Wilcoxon rank sum and chi-square tests as appropriate. The Fisher exact test was used to test for a genetic interaction between BAP1 and PBRM1. Cox models and the HR with the 95% CI were used to assess the association of PBRM1 and BAP1 expression (dichotomized as negative vs positive) with the risk of death from RCC after adjusting for patient age. We also tested for PBRM1 by BAP1 interaction in the Cox models after adjusting for corresponding main effects and age. The Kaplan-Meier method was used to estimate time to RCC specific death. Patients without RCC specific death were censored at death or last followup. Statistical analysis was performed with R, version 2.15 (<https://www.r-project.org/>). All tests were 2-sided with $p < 0.05$ considered statistically significant.

RESULTS

Impact of PBRM1 on Demographic, Pathological and Clinical Outcomes

Staining failed in 36 of 1,479 ccRCCs (2.4%), 28 (1.9%) had diffusely weak staining and 80 (5.4%) had heterogeneous staining (supplementary fig. 1, <http://jurology.com/>). After removing these tumors and those without clinical data (0.3%) we were left with 1,330 samples, representing 90% of the initial cohort of 1,479. Of these samples 656 (49.3%) did and 674 (50.7%) did not express PBRM1. PBRM1 deficient tumors were found in older patients and showed slightly worse tumor size, grade and invasion (supplementary table 1, <http://jurology.com/>). After adjusting for age PBRM1– tumors carried a higher risk of metastasis (HR 1.46, $p = 0.0011$). However, unlike in prior studies^{12,13} we did not observe an increased risk of death from RCC (HR 1.083, $p = 0.54$, table 1 and fig. 1).

PBRM1 and BAP1

Subject to Negative Genetic Interaction—We integrated the results of PBRM1 expression with recent results of BAP1 expression in the same tumor cohort.¹¹ Of the 1,330 tumors that stained positive or negative for PBRM1 85% also stained positive or negative for BAP1, leaving 1,258 available for primary analysis (supplementary fig. 2, <http://jurology.com/>). Using IHC we were able to specifically focus on tumors with simultaneous loss of PBRM1 and BAP1, and distinguish them from those in which PBRM1 and BAP1 were lost in different tumor cell populations (fig. 2).

Given the frequency of BAP1 and PBRM1 negative tumors in the individual total cohorts and under assumptions of independence, we would have expected that 5.3% ($10.6\% \times 50.4\%$) of tumors would be PBRM1– BAP1–. However, the frequency of PBRM1– BAP1– tumors was 1.8%. The odds of BAP1 loss in PBRM1 deficient tumors were approximately a fifth of the odds of BAP1 loss in PBRM1 expressing tumors (Fisher exact test OR, 0.18, 95% CI 0.11–0.28, $p < 0.00001$). These data provide evidence of negative selection of PBRM1– BAP1– tumors consistent with negative genetic interaction between PBRM1 and BAP1. This demonstrates the importance of considering both genes/proteins simultaneously in analyses.

Combined Analyses Defined 4 Biological Subtypes of ccRCC with Stepwise Changes in Aggressiveness and RCC Specific Survival—We divided tumors into 4 subtypes based on PBRM1/BAP1 expression, including 1) PBRM1+ BAP1+ in 514 cases (40.9%), 2) PBRM1– BAP1+ in 611 (48.6%), 3) PBRM1+ BAP1– in 110 (8.7%) and 4) PBRM1– BAP1– in 23 (1.8%). We observed progressive stepwise worsening in tumor size, nuclear grade, necrosis and TNM stage from PBRM1+ BAP1+ to PBRM1– BAP1+ to PBRM1+ BAP1– to PBRM1– BAP1– (table 2). In particular PBRM1– BAP1– tumors were associated with aggressive features. Of those lesions 91% were high grade and 57% showed necrosis.

Stepwise worsening in clinical outcomes also existed among subtypes (fig. 3). Ten-year RFS was 83.6% for PBRM1+ BAP1+, 69.2% for PBRM1– BAP1+, 58.4% for PBRM1+ BAP1– and 45.7% for PBRM1– BAP1–. Compared to wild-type ccRCC the HR of RFS was 1.913 (95% CI 1.46–2.507, $p = 2.54E-06$) for PBRM1– BAP1+, 3.159 (95% CI 2.178–4.581, $p = 1.33E-09$) for PBRM1+ BAP1– and 4.939 (95% CI 2.726–8.948, $p = 1.38E-07$) for PBRM1– BAP1–. After adjusting for the SSIGN score only the PBRM1– BAP1+ cohort remained with significantly worse RFS (HR 1.543, 95% CI 1.125–2.117, $p = 0.007$, table 1).

Ten-year estimated RCC specific survival was 86.7% for PBRM1+ BAP1+, 79.7% for PBRM1– BAP1+, 59.9% for PBRM1+ BAP1– and 42.4% for PBRM1– BAP1–. Compared to wild-type ccRCC the HR of RCC specific death was 1.394 (95% CI 1.024–1.898, $p = 0.03491$) for PBRM1– BAP1+, 3.251 (95% CI 2.177–4.856, $p = 8.4 \times 10^{-9}$) for PBRM1+ BAP1– and 5.2 (95% CI 2.847–9.563, $p = 9.1 \times 10^{-8}$) for PBRM1– BAP1–. After adjusting for the SSIGN score no cohort had a significantly different HR for the risk of RCC specific survival (table 1).

Analysis Including Weak Positive and Focal Negative Findings

As an exploratory analysis we included samples that were not uniformly positive or negative (focal negative and weak positive) for PBRM1 and BAP1. As explained we grouped tumors that were focally negative with negative tumors and those that were weakly positive with positive tumors. Reassuringly the overall result of this grouping remained similar (supplementary fig. 3, <http://jurology.com/>).

DISCUSSION

We present a biological classification of ccRCC based on BAP1 and PBRM1 expression. We found that these proteins are interrelated and simultaneous loss of both proteins occurs at a frequency lower than that expected by chance alone, consistent with a negative genetic interaction. Given this negative interaction we conclude that the clinical significance of PBRM1 and BAP1 should be determined in combined rather than univariate fashion. Based on this conclusion we found that combined expression of PBRM1 and BAP1 identified 4 groups of patients with differing demographic, pathological and cancer specific outcomes. Specifically patients with PBRM1+ BAP1+ tumors had the best clinical outcomes and patients with PBRM1– BAP1+, PBRM1+ BAP1– and PBRM1– BAP1– tumors had sequentially worse outcomes. This classification stands out from other classifications in that it is based on tumor driving events rather than on epiphenomenology biomarkers with unclear links to tumor biology. Furthermore, because of the location on chromosome 3p and the fact that loss of chromosome 3p is a truncal event,¹⁴ the *BAP1* and *PBRM1* genes are likely to be inactivated early during tumor evolution.¹⁵

In comparison to previous studies of the clinical significance of PBRM1 expression our series has several key differences. The 2 previous studies evaluating PBRM1 expression by IHC concluded that PBRM1 deficient tumors were associated with worse outcomes.^{12,13} da Costa et al tested 112 ccRCCs and determined that PBRM1 deficient tumors were associated with decreased disease-free survival ($p = 0.017$).¹³ Pawlowski et al evaluated 227 ccRCCs and reported that PBRM1 deficient tumors were associated with decreased overall survival ($p = 0.025$).¹² However, we did not observe a difference in RCC specific survival when tumors were classified simply based on PBRM1 status. Both prior studies were relatively small with fewer than 250 patients, involved tissue microarrays, had marginal p values and did not assess for BAP1 in the same cohort.^{12,13} When we removed BAP1 deficient tumors, we found a difference in RCC specific survival between PBRM1+ BAP1+ and PBRM1– BAP1+ tumors. However, the difference was small (HR 1.394, 95% CI 1.024–1.898) and the p value was marginal ($p = 0.03491$).

An important contribution of our study involves further defining the relationship between PBRM1 and BAP1 in ccRCC. The underrepresentation of doubly mutated tumors strongly suggests a negative genetic interaction. Multiple definitions exist of genetic interaction¹⁶ and we use the term to refer to interdependence between 2 genes that affects the observed frequency of concurrent mutations. A prior study in 176 tumors showed that 3 had mutations in *BAP1* and *PBRM1* while 13 had been expected.⁸ In a meta-analysis of 3 other studies 6 tumors were found with mutations in *BAP1* and *PBRM1* while 14 had been expected.¹⁷

Given the low frequency of PBRM1 and BAP1 loss in tumors, a large series was required to characterize this interaction. In our series of greater than 1,000 patients we identified 23 tumors that were simultaneously deficient for PBRM1 and BAP1. These tumors were associated with the most aggressive pathological features and a median estimated RCC specific survival of 7.7 years. The estimated 15-year RCC survival rate approached 0. In contrast the estimated 15-year RCC survival rate in patients with PBRM1+ BAP1+ tumors was greater than 80%. Thus, despite the low frequency it is important to identify these patients so that they may benefit from more frequent followup or adjuvant interventions.

What causes the underrepresentation of tumors simultaneously deficient for BAP1 and PBRM1 is unknown. PBRM1 and BAP1 are nuclear proteins implicated in chromatin remodeling and regulation of gene expression.¹⁰ PBRM1 is the targeting subunit of a nucleosome remodeling complex and BAP1 is a deubiquitinase that regulates the ubiquitination of histone H2A (H2AK119ub1) among others.¹⁵ We speculate that alterations induced by the simultaneous loss of both proteins may not synergize in tumorigenesis except in some permissive contexts. Finally and perhaps most importantly, despite the unknown biological rationale of the genetic interaction between PBRM1 and BAP1 we conclude that these data support the simultaneous analysis of PBRM1 and BAP1.

Multiple groups, including ours, have reported that additional biomarkers can improve the prognostic significance of conventional algorithms such as the SSIGN score.^{9,18–20} However, in the current study PBRM1 and BAP1 expression did not add predictive information to the SSIGN score. Despite the lack of prognostic significance we believe that PBRM1 and BAP1 expression remain important for several reasons. 1) *PBRM1* and *BAP1* are two of the most commonly mutated genes in ccRCC. 2) These mutations are proximal truncal mutations that may influence tumor evolution. 3) Their status defines 4 subtypes of ccRCC that differ in the likelihood of recurrence. 4) These subtypes show different gene expression patterns (and biology) and may respond differently to therapy. 5) Identifying targets downstream will pave the way for subtype specific therapies. Overall PBRM1 and BAP1 offer the opportunity for a biologically based classification of ccRCC.

There are several limitations of our study. 1) SETD2, another gene that is mutated at a frequency of greater than 10% in ccRCC, is not considered in this classification. 2) Multiple additional driver genes are mutated in ccRCC, although most other genes are mutated at a frequency of less than 5%. 3) We excluded a small cohort of samples from our analysis due to failed staining or equivocal PBRM1/BAP1 staining. We compared demographics and RCC related outcomes in the included vs excluded cohorts and found several significant differences between the groups (supplementary table 2, <http://jurology.com/>). Excluded patients had significantly higher grade tumors and a higher percent of tumor necrosis. Whether tumor grade or necrosis impacted the IHC assay to assess PBRM1 and BAP1 remains unclear. Therefore, we interrogated the entire cohort in a secondary analysis and reassuringly found that the results did not change significantly. 4) Our study only included 2 representative slides (1 for PBRM1 and 1 for BAP1) per tumor. Given the molecular heterogeneity of ccRCC, it is possible that some tumors had different PBRM1 and BAP1 status depending on the block.

CONCLUSIONS

We report a biological classification of ccRCC based on PBRM1 and BAP1 protein expression that impacts patient outcomes. *PBRM1* and *BAP1* are 2 of the most commonly mutated genes in ccRCC and they are mostly mutually exclusive. The strong associations of *PBRM1* and *BAP1* expression with pathological and clinical outcomes further emphasize the biological importance of these genes for classifying ccRCC. Ongoing investigation is needed to determine the clinical significance of additional mutations in ccRCC and whether PBRM1 or BAP1 expression is associated with the response to currently available therapies for ccRCC.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations and Acronyms

BAP1	BRCA associated protein 1
ccRCC	clear cell RCC
IHC	immunohistochemistry
PBRM1	polybromo 1
RCC	renal cell carcinoma
RFS	relapse-free survival

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Figure 1.

RCC specific outcomes in patients by PBRM1 expression. *A*, patients with PBRM1– tumors were at increased risk for metastasis vs patients with PBRM1+ tumors (HR 1.46, $p = 0.001$). *B*, after adjusting for age patients with PBRM1– tumors were not at increased risk for RCC death vs patients with PBRM1+ tumors (HR 1.08, $p = 0.54$).

Figure 2.

IHC reveals molecular heterogeneity of PBRM1 and BAP1 in same ccRCC sample. Positive stained nuclei in BAP1- or PBRM1- tumors correspond to stromal or inflammatory cells. These nuclei were typically smaller than tumor nuclei and showed more condensed chromatin. *A*, tumor with 2 areas, including 1 BAP1+ PBRM1- area and 1 BAP1- PBRM1+ area. *B*, tumor with loss of BAP1 and PBRM1 throughout. Scale bars indicate 100 μm. Insets, reduced from X400.

Figure 3.

RCC specific outcomes in patients by PBRM1 and BAP1 protein expression. PBRM1+ BAP1+ served as HR referent. *A*, metastasis risk significantly differed among 4 cohorts. For PBRM1- BAP1+ HR 1.91, for PBRM1+ BAP1- HR 3.16 and for PBRM1- BAP1- HR 4.94 (each $p < 0.00001$). *B*, risk of RCC death significantly differed among 4 cohorts. For PBRM1- BAP1+ HR 1.39 ($p = 0.03$), for PBRM1+ BAP1+ HR 3.25 ($p < 0.00001$) and for PBRM1- BAP1- HR 5.22 ($p < 0.00001$).

Table 1

PBRM1 and BAP1 expression by RCC related outcomes

Adjustment	RCC Death Risk		Metastasis Risk		
<i>PBRM1 in 1,330 tumors</i>					
Age:	HR (95% CI)		p Value		
PBRM1+	1.0	(referent)	–	1.0	(referent)
PBRM1–	1.083 (0.839–1.398)		0.54	1.457 (1.163–1.827)	
Age + SSIGN score:					
PBRM1 +	1.0	(referent)	–	1.0	(referent)
PBRM1–	1.054 (0.787–1.412)		0.72	1.501 (1.153–1.953)	
<i>BAP1 + PBRM1 in 1,258 tumors</i>					
Age:					
PBRM1+ BAP1+	1.0	(referent)	–	1.0	(referent)
PBRM1– BAP1+	1.394 (1.024–1.898)		0.03491	1.913 (1.46–2.507)	
PBRM1+ BAP1–	3.251 (2.177–4.856)		8.41E-09	3.159 (2.178–4.581)	
PBRM1– BAP1–	5.217 (2.847–9.563)		9.10E-08	4.939 (2.726–8.948)	
Age + SSIGN score:					
PBRM1+ BAP1+	1.0	(referent)	–	1.0	(referent)
PBRM1– BAP1+	1.032 (0.725–1.47)		0.8611	1.543 (1.125–2.117)	
PBRM1+ BAP1–	0.94 (0.582–1.52)		0.8016	1.094 (0.699–1.712)	
PBRM1– BAP1–	1.136 (0.527–2.446)		0.7453	1.141 (0.535–2.435)	

Table 2
Clinical and pathological information on patients in 4 subgroups defined by BAP1 and PBRM1

	Overall	BAP1+ PBRM1+	BAP1+ PBRM1-	BAP1- PBRM1+	BAP1- PBRM1-	p Value
No. pts (%)	1,258	514	611	110	23	0.69
No. female (%)	435	185 (34.6)	207 (36.0)	34 (33.9)	9 (39.1)	
No. male (%)	823	329 (65.4)	404 (64.0)	76 (66.1)	14 (60.9)	
Mean/median age at surgery (range)	63.1/64.2 (19.8–90.2)	60.0/60.6 (19.8–90.2)	65.5/66.1 (35.7–88.2)	63.3/63.5 (35.9–90.0)	68.3/70.1 (41.1–84.7)	<0.0001*
Mean/median cm tumor size (range)	6.0/5.0 (0.5–29.0)	5.3/4.0 (0.5–24.0)	6.2/5.4 (0.8–22.0)	8.2/7.9 (1.4–29.0)	8.8/9.3 (3.5–15.0)	<0.0001*
No. TNM stage (%):						<0.0001
Missing	4	1	3	0	0	
1	772	369 (61.6)	356 (71.9)	42 (58.6)	5 (21.7)	
2	175	64 (14.0)	86 (12.5)	19 (14.1)	6 (26.1)	
3	297	79 (23.7)	160 (15.4)	47 (26.3)	11 (47.8)	
4	10	1 (0.8)	6 (0.2)	2 (1.0)	1 (4.3)	
No. nuclear grade (%):						<0.0001
1	104	63 (8.3)	41 (12.3)	0 (6.7)	0	
2	574	283 (45.6)	281 (55.1)	8 (46.0)	2 (8.7)	
3	508	151 (40.4)	258 (29.4)	81 (42.2)	18 (78.3)	
4	72	17 (5.7)	31 (3.3)	21 (5.1)	3 (13.0)	
No. coagulative tumor necrosis (%):						<0.0001
No	993	435 (78.9)	489 (84.6)	59 (80.0)	10 (43.5)	
Yes	265	79 (21.1)	122 (15.4)	51 (20.0)	13 (56.5)	
No. SSIGN category (%):						<0.0001
Missing	189	68	92	21	8	
0–3	760	362 (71.1)	356 (81.2)	39 (68.6)	3 (43.8)	
4–7	244	71 (22.8)	135 (15.9)	29 (26.0)	9 (32.6)	
8+	65	13 (6.1)	28 (2.9)	21 (5.4)	3 (23.6)	

* Wilcoxon rank test.