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Genomic Heterogeneity and the Small Renal Mass

Daiki Ueno¹, Zuoquan Xie^{1,2}, Marta Boeke¹, Jamil Syed¹, Kevin A. Nguyen¹, Patrick McGillivray¹, Adebawale Adeniran³, Peter Humphrey³, Garrett M. Dancik⁴, Yuval Kluger³, Zongzhi Liu³, Harriet Kluger⁵, and Brian Shuch¹

¹Departments of Urology, Yale School of Medicine, New Haven, Connecticut

²Division of Antitumor Pharmacology, State Key Laboratory of Drug Research, Shanghai Institute of Materia Medica, Chinese Academy of Sciences, Shanghai, China

³Departments of Pathology, Yale School of Medicine, New Haven, Connecticut

⁴Computer Science Department, Eastern Connecticut University, Willmantic, Connecticut

⁵Departments of Medical Oncology, Yale School of Medicine, New Haven, Connecticut

Abstract

Purpose—Tumor heterogeneity may represent a barrier to pre-operative genomic characterization by needle biopsy in clear cell renal cell carcinoma (ccRCC). The extent of heterogeneity in small renal tumors remains unknown. Therefore, we set out to evaluate heterogeneity in resected large and small renal tumors.

Experimental Design—We conducted a study from 2013 through 2016, that evaluated 47 consecutive ccRCC tumors resected during radical or partial nephrectomy. Cases were designated as small (<4cm) and large (>7cm) tumors. Each tumor had 3 regions sampled. Copy number variation (CNV) was assessed and Gene expression analysis was performed to characterize the clear-cell A and B (ccA/ccB) profile and the Cell Cycle Progression (CCP) score. Genomic heterogeneity between 3 regions was evaluated using CNV subclonal events, regional expression profiles, and correlation between gene expression.

Results—23 small and 24 large tumors were analyzed. Total CNVs and subclonal CNVs events were less frequent in small tumors ($p < 0.001$). Significant gene expression heterogeneity was observed for both CCP scores and ccA/ccB classifications. Larger tumors had more variance in

Corresponding Author: Brian Shuch, Department of Urology, Yale School of Medicine, PO Box 208058 New Haven, CT 06520-8058, T 203-785-2815, F 203-785-6475, Brian.Shuch@yale.edu.

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The authors declare no potential conflicts of interest.

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Author Contributions

Conception and design: Daiki Ueno, Xie Zuoquan, Marta Boeke, Brian Shuch

Development of methodology: Daiki Ueno, Xie Zuoquan, Marta Boeke, Brian Shuch

Acquisition of data: Daiki Ueno, Xie Zuoquan, Marta Boeke, Brian Shuch

Analysis and interpretation of data: All authors

Writing, review and/or revision of the manuscript: Daiki Ueno, Brian Shuch

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Study supervision: Brian Shuch

CCP scores ($p=0.026$). The distribution of ccA/ccB differed between small and large tumors with mixed ccA/ccB tumors occurring more frequently in the larger tumors ($p=0.024$). Analysis of 5 mixed tumors (with both ccA/ccB regions) demonstrated the more aggressive ccB phenotype had greater CNV events ($p=0.014$).

Conclusions—Small renal tumors have much less genomic complexity and fewer subclonal events. Pre-treatment genomic characterization with single needle biopsy in small tumors may be useful to assess biologic potential and may influence therapy.

Keywords

Small Renal Mass; Renal Biopsy; Genomic Profile; Renal Cell Carcinoma; Heterogeneity

Introduction

The past three decades have seen a dramatic increase in the incidence of renal cell carcinoma (RCC) largely attributed to the increased rate of cross-sectional imaging. The rising incidence of RCC has largely been due to small renal masses (SRM) ≤ 4 cm with most of them asymptomatic at diagnosis.¹ With the changing pattern of detection, now approximately 60% of new diagnoses are stage I RCC.² The clinical outcome in patients with early stage RCC is generally excellent when treated surgically, although approximately 25% of high-grade stage I RCCs still metastasize.³ However the relatively stable mortality rate, despite the increased incidence, has suggested overdiagnosis and overtreatment.⁴ Overtreatment may pose significant health risks, as the majority of kidney cancer patients are elderly and/or have preexisting comorbidities. Besides the immediate morbidity of treatment, overtreatment may pose long-term adverse health outcomes as there are increased renal and cardiovascular events associated with all forms of therapy.⁵

For elderly individuals who are not candidates for treatment, non-operative management led to the recognition that many SRM exhibit a low growth rate.^{6,7} In recent years the concept of active surveillance has become more widely accepted.⁸ Despite the safety, the adoption of this approach has been low and is currently implemented in approximately 10% of new diagnoses.⁹ Despite the acceptance of this treatment modality by the American Urologic Association (AUA),¹⁰ a significant barrier exists in the widespread adoption of active surveillance as there are no reliable methods to determine biologic potential.

The introduction of cancer genomics to clinical practice has revolutionized the diagnostic, prognostic, and therapeutic approach in various malignancies. In kidney cancer, the molecular events associated with clear cell RCC (ccRCC) have been characterized due to large-scale genomic sequencing efforts such as the Cancer Genome Atlas (TCGA). Similarly, transcriptomic profiling has identified specific clusters of tumors with worse behavior after nephrectomy, which may be useful in the study of SRM prior to treatment. The clear cell A and B (ccA/ccB) designation and the Cell Cycle Progression (CCP) Score are two mRNA classifiers that may be useful in the evaluation of recurrence-free and overall survival and are being further developed for clinical care.^{11,12}

While genomic alterations can be reliably detected from a single renal tumor biopsy, the impact of precision medicine has unfortunately been limited. One of the perceived obstacles to this approach is the concern of significant tumor heterogeneity. Previous studies of heterogeneity in RCC tumors evaluated only large and metastatic kidney tumors.^{13,14} In these tumors, approximately 75% of driver alterations (copy number and mutations) were found to be subclonal, i.e. not shared throughout all tumor regions. Thus a single biopsy may not sufficiently identify particular genetic events that may be relevant to clinical management. While it may be safe to perform multi-site biopsies within a large primary tumor, this is not feasible in a SRM in which obtaining sufficient tissue on a single biopsy can be challenging. While heterogeneity may limit genomic characterization from a single biopsy in the large renal mass, its clinical relevance is currently unknown in the SRM that may be eligible for active surveillance.

In order to define the feasibility of pre-operative genomic characterization of the SRM, we set out to compare the extent of genomic heterogeneity in a consecutive series of resected small and large ccRCC tumors. We evaluated subclonal events involving driver copy number variations (CNV) and determined prognostic gene expression signatures to better define the limitation of a single region biopsy.

Materials and Methods

Research Subjects and Specimen Processing

All patients undergoing surgery were provided with informed consent and offered enrollment into a biospecimen repository approved by a Yale University Institutional Review Board. Between 2013 and 2016, 100 consecutive research subjects undergoing radical or partial nephrectomy had tumor procurement. Prior to resection, the surgeon denoted clinical stage of the tumor and cases were designated as small (<4cm, cT1a) and large (>7cm, cT2+) renal tumors. Intermediate sized tumors (4–7cm) were excluded from further analyses. Research material was procured within 30 minutes of removal and processed in accordance with institutional and College of American Pathology guidelines. Upon tumor sectioning, clinical pathology assistants sampled non-necrotic components 1 cm apart and designated regions 1–3 (R1–R3). Tumors were snap frozen with liquid nitrogen. Members of the genitourinary pathology team (AA, PH) reviewed all cases and those determined to be ccRCCs were selected for our analysis. The highest Fuhrman grade was assigned and individual regions did not take specific regional grade assessment. DNA and RNA were extracted from individual regions using a Maxwell-16 SimplyRNA Tissue Kit, and Maxwell-16 DNA Purification Kit (Promega, Madison, WI). Nucleic acids were quantified using a Nanodrop 2000 (ThermoFisher, Wilmington, DE).

Analysis of Copy Number

DNA samples were applied to an Illumina CytoSNP 12 bead array (Illumina, San Diego, CA), using a total of 500 ng input. Fifteen recurrent CNVs identified in TCGA project were selected for review. Chromosomal locations included 11 regions of loss (3p25, 14q24, 9p21.3, 6q26, 8p11, 10q23, 1p36, 4q35, 13q21, 15q21, and 2q37) and 4 regions of gain (5q35, 8q24, 3q26, and 1q32). CNVs were assessed using Blue Fuse Genomic Studio

(Illumina) and manually confirmed by inspection of the LogR ratio and B allele frequency. We defined CNVs as being present in an individual tumor region if $\geq 15\%$ of the sample had evidence of a gain or loss.²⁰ The presence of subclonal/branch events was assessed only when 3 regions had successful profiling and was given this term when an alteration was not shared among all regions. For TCGA samples, CNV data was downloaded from cbioportal.org and analyzed using the Integrated Genomic Viewer (IGV, version 2.3.72).¹⁵

Nanostring and data analysis of classification ccA/ccB and CCP Score

Gene expression profiling was performed on 200ng of RNA using a custom designed hybridization probe set to evaluate 34 genes involved in designation of ccA/ccB and 31 genes involved in calculation of the CCP score.^{11,16} We included the same house keeping genes (5 and 15 for ccA/ccB and CCP scores, respectively) used in the development of both classification systems. Gene expression analysis was performed using the Nanostring nCounter system for digital RT-PCR and analyzed with nSolverAnalysis Software. (NanoString Technologies, Seattle, WA). A previously described prediction analysis of microarrays (PAM) classifier was applied to the normalized microarray data for designation of ccA/ccB using R version 3.3.0.¹² While the CCP score calculation was initially designed using qPCR, it has successfully been calculated using microarray data.¹⁷ And in a similar way, we calculated the CCP score using Nanostring data. Briefly, after normalization with R package DESeq2 and log2 transformation, the gene expression was z-normalized across samples, and a CCP score was created for each sample as the mean expression value of the normalized CCP genes.

Statistical Analysis

For the purpose of determining sample size, we hypothesized that subclonal CNV events were three times more common in larger tumors. Using an 80% power and $p = 0.05$, we determined we needed a cohort of 20 small and 20 large tumors to show a three-fold increase in subclonal CNV events for larger tumors. Continuous variables were compared using the independent t test. Chi squared or Fisher's exact tests were used to evaluate differences in categorical variables. Non-parametric testing of continuous variables was performed using the Mann-Whitney U test. Correlation between continuous variables was determined using linear regression. Intra-tumoral correlation of gene expression was evaluated by individual and combined gene sets for each tumor region using principal component analysis and heatmaps. The intra-tumoral correlation was determined using the Spearman correlation. The determination whether the distributions of correlation coefficients between large and small samples differ significantly was evaluated using the Kolmogorov-Smirnov test. Statistical significance was taken at $p = 0.05$ for all comparisons. When multiple hypotheses were tested, the false discovery rate (FDR) correction was applied. Statistical calculations were performed using R V3.4 (R Core Team), Matlab V2014b (The MathWorks, Inc.), SPSS V24 (IBM).

Results

A total of 23 small and 24 large renal tumors were included in the study. Subject and tumor characteristics are shown in Table 1. Small tumors were more frequently stage T1 (95.7%

[22 of 23]) and a lower Fuhrman grade (95.7% [22 of 23] grade 1 and 2). Large tumors were higher grade (79.1% [19 of 24] grade 3+), higher stage (79.1% [19 of 24] T3+), and more frequently had necrotic areas (62.5% [15 of 24]) ($p < 0.001$, all associations).

CNVs were observed with similar frequency to previous reports, with all known recurrent alterations present in 5 tumors.¹⁸ 22 large and 22 small tumors were analyzed for CNVs, 43 (97.7%) had 3p25 loss. The other common findings (Figure 1a) were 5q35 gain (27 [61.4%]), 14q24 (22 [50%]), 8p11 (18 [40.9%]), 1p36 (17 [38.6%]), and 10q23 (16 [36.4%]) losses. When comparing CNVs in large versus small tumors, there was a significant increase in 14q24 loss in large tumors (17 [77.3%] vs 5 [22.7%], $p < 0.001$) (Figure 1b). An increase in 9p21.3 (10 [45.5%] vs 2 [9.1%], $p = 0.007$), 8p11 (13 [59.1%] vs 5 [22.7%], $p = 0.014$), 2q37 (5 [22.7%] vs 0 [0%], $p = 0.048$) losses and 3q26 (6 [27.3%] vs 0 [0%], $p = 0.021$) gain were observed in large tumors, however there were not statistically significant after multiple hypothesis testing.

Analysis of multi-region CNVs demonstrated a greater burden of CNV events for large tumors (median [range], 7.0 [2–11] vs 2.5 [1–10], $p < 0.001$) (Figure 2a). A similar analysis within TCGA (using one region) demonstrated similar results with large tumors having more CNV events (median [range], 4 [0–13] vs 2 [0–11], $p < 0.001$) (Figure 2b). We noted subclonal CNVs were more frequent in large tumors (median [range], 4 [0–10] vs 0 [0–8], $p < 0.001$) (Figure 2c). For each particular CNV, the prevalence of that CNVs among subclones was measured, CNV events occurring at 3p25, 5q35, 6q26, 15q21, and 2q37 appeared to be shared/truncal events as when present, they occurred in 50% of cases (Figure 1c). 13q21, 1q32, 8q24, 3q26 were likely subclonal/branch events as they were shared among all regions in less than 25% of cases.

In 21 small tumors and 21 large tumors, ccA/ccB classification was determined for individual regions and by tumor. Overall, 100 regions were ccA and 26 regions were ccB. The distribution of ccA/ccB differed between small and large tumors with mixed ccA/ccB tumors occurring more frequently in the larger tumors (Figure 3, $p = 0.024$). Of 21 small tumors, 19 were classified as ccA, 1 as ccB, and 1 as mixed (a tumor consisting both ccA and ccB regions) designation. Of 21 large tumors, 11 were classified as ccA, 5 as ccB, and 5 as mixed designation. For the 6 mixed tumors, 5 tumors (15 regions) had available CNV data for comparison between ccA and ccB regions. CNVs were more frequent among ccB (median [range], 7 [2–8] vs 2 [0–6], $p = 0.014$) (Supplementary Fig. S1).

Median CCP scores did not differ between small and large tumors. However, there was increased variance ($p = 0.026$) for large tumors. Gene expression of both prognostic gene sets showed significant within-sample variation suggested by principal component analysis (PCA) plots (Supplementary Fig. S2) and heat maps (entire gene set, Supplementary Fig. S3). Spearman correlation coefficients for the entire gene set between regions ranged from 0.6–0.97. Evaluation of the minimal correlation between regions by size category showed that there was significantly more intratumoral heterogeneity in large tumors (Figure 4, $p = 0.004$ Kolmogorov-Smirnov test). Within the entire cohort, we found decreasing gene expression correlation with increasing subclonal CNV events ($R^2 = 0.431$, $p < 0.001$) (Supplementary Fig. S4).

Discussion

A pre-treatment renal mass biopsy may provide pathologic characteristics that could be useful to select patients with increased surgical risk for active surveillance. With current techniques, a renal mass biopsy provides a high diagnostic rate with minimal morbidity.¹⁹ However the accuracy of grading on biopsy is not perfect with 50–85% accuracy likely limited by significant grade heterogeneity.^{20,21} Therefore, many providers do not routinely biopsy since the results rarely alter anticipated treatment. Despite limitations on pathologic diagnosis, a biopsy provides access to tissue for genomic characterization. However, until recently there were limited testing options due to costs, limited material, difficulty with genomic studies on fixed tissue, and long processing times.

In recent years the widespread availability and reduced costs of genomic testing have led to increased clinical utilization. In a wide variety of malignancies, testing is accepted as a useful tool in determining the risk of recurrence, prognosis, the utility of adjuvant therapy/radiation, and the selection of molecular targeted therapy. For localized disease, various genomic tests have been introduced. Some of these assays define the likelihood of higher risk features or adverse outcomes at the time of surgery in order to better counsel those interested in active surveillance- something that could also be useful in patients presenting with a SRM. There has been extensive characterization of the recurrent driver alterations associated with the various histologic subtypes based on work from TCGA. Despite the recognition of various CNVs and somatic mutations associated with worse outcome in ccRCC, genomic characterization has not made its way into established clinical guidelines.²²

One of the major obstacles to adoption is the perception of massive intra-tumor heterogeneity, a perception that is based on the study of advanced kidney cancer.^{13,14} Whether this is due to larger tumors being present for longer resulting in an increased number of cell divisions or whether these larger tumors are predisposed to being more aggressive has yet to be determined. How genomic heterogeneity relates to the SRM is critical to understanding how genomic characterization from a single biopsy may guide important clinical decisions for such as selection for surveillance, utility of node dissection, and selection for nephron-sparing surgery.

We set out to characterize genomic heterogeneity in the small renal mass (cT1a, ≤4 cm) and large renal masses (cT2+, >7 cm) as a comparison to the types of tumors evaluated in similar studies.^{13,14} This cohort (n=47) currently is the largest multisite assessment of tumor heterogeneity in ccRCC and utilized both CNV and mRNA gene expression. Our major finding from CNV analysis is that small renal tumors have significantly less overall genomic events and that subclonal gains/losses are much less frequent in these tumors. Thus the homogeneous nature of the SRM, may allow sufficient characterization of known adverse CNVs from one or two sites. While the sensitivity and specificity for an assay based on a single core biopsy may not be 100%, a clinician may be able to better counsel a patient on having a tumor with more aggressive potential and strongly consider treatment versus a more stringent active surveillance protocol.

Our assessment of subclonal CNVs identified significant differences in the frequency of shared events, likely supporting the temporal order of genomic events that occur during tumor evolution. The high incidence of shared CNVs at 3p25, 5q35, 6q26, 15q21, and 2q37 suggest these events occur early in tumorigenesis and are more likely truncal events. Gerlinger and colleagues similarly found differences in the frequency of shared CNVs, however, important differences were observed.^{13,14} While 3p and 5q CNVs were also found to be shared events, losses of 4q, 8p, and 14q were also believed to be early events. We also found that several CNVs were infrequently shared, suggesting these events occur as branch events that occur late in tumorigenesis. These included 13q21 loss and 1q32, 8q24, and 3q26 gains. Similar issues have been raised with mutational profiling in a cohort of 14 ccRCC tumors reported by Sankin.²³ In that study, clear differences in the ubiquitous nature of the major driver mutations were observed similar to our findings.

In recent years there have been efforts to apply transcriptomic profiling in RCC, mostly those with locally advanced or metastatic disease. The ccA/ccB classification has been applied to large cohorts of patients with metastatic disease demonstrating the utility at determining prognosis and prediction of response to sunitinib.^{24,25} We performed heterogeneity assessment of genes used in these promising transcriptomic classifiers, including the ccA/ccB system and the CCP score. Transcriptomic RNA classifiers have been considered an attractive option for prognostic models in light of the heterogeneous nature of driver alterations. Global expression may be less sensitive to individual genomic events. However, we demonstrate that heterogeneity of the transcriptomic profile can occur within various tumor regions, especially for large renal masses, which have a mixed pattern in nearly a quarter of tumors. This finding differs from a report by Serie and colleagues, who observed no classification heterogeneity within the resected primary tumor, implying that a single sample per tumor should be sufficient in classification.²⁶ However, similar to the more homogenous nature of CNVs in small renal masses, we found that small renal tumors had a more consistent ccA/ccB classification with only 1 of 21 tumors having a mixed profile. Larger tumors also had increase variance in the CCP score which may need to be taken in context when further evaluating this emerging prognostic marker that has been recently validated in two institutional cohorts.²⁷ Our comparison of transcriptomic profiling similarly shows significantly less heterogeneity for small tumors with nearly all tumors having 90% correlation between regions.

The heterogeneity of gene expression has limitations for advanced disease characterization likely related to divergence of clonal populations. For our mixed samples, we demonstrate that CNV events are more common in ccB regions. This could suggest that as tumors grow and evolve, the acquisition of late or subclonal chromosomal changes leads to divergence of gene expression. Analysis of multiregion sampling of metastatic samples in the Gerlinger cohort found that 80% of the tumors contained mixed gene expression profiles of ccA/ccB.^{14,28} The more aggressive ccB regions had increased numbers of poor prognostic somatic mutations and CNVs. Both of these findings suggest that increased genomic instability may be driving a change in tumor classification. This is also supported by the Serie cohort, which found that among mixed metastatic samples that underwent longitudinally sampled, 80% of cases progressed to the ccB phenotype.²⁶ Thus RNA profiling, while perhaps useful for a single biopsy in a SRM, may require multisite biopsies and assessment in larger tumors to

improve the sensitivity of detection of the ccB phenotype. In the future, characterization of overall, intratumoral genomic heterogeneity from a single biopsy may be incredibly useful prior to inferring the genomic profile from a single site.

This study has several limitations based on the methodology. Our work is based on a moderate-size cohort (n=47) and an expanded cohort could improve conclusions and improve the statistical comparisons between groups. Nearly 95% of our SRMs were low grade, and this limits conclusions about the rare high grade tumor. *Ex vivo* sampling at the time of radical or partial nephrectomy is different from percutaneous biopsy since it is not done at “random” and we did not microdissect the most representative area of the tumor which is often done with tumor profiling.

Conclusion

We demonstrate that SRM may be eligible for active surveillance have limited CNV and transcriptomic heterogeneity. Our findings temper some of the concerns that a pretreatment biopsy and characterization cannot overcome massive genomic heterogeneity in ccRCC. While this may be true in advanced disease and large tumors, it appears less relevant to the majority of small tumors, which are often those detected incidentally. This type of approach could decrease the morbidity associated with surgical management. Further analyses will be useful to confirm our findings and evaluate other RCC subtypes and tumors between 4–7 cm in addition to the evaluation of heterogeneity driver mutations and other mRNA prognostic genes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Translational Relevance

Tumor heterogeneity is believed to represent a barrier to pre-operative genomic characterization in kidney cancer. Previous studies of heterogeneity in clear cell renal cell carcinoma (ccRCC) evaluated only large and metastatic tumors. In small renal tumors in which multiple biopsies are not feasible, the extent of heterogeneity remains unknown. In this study, we evaluated how the extent of genomic heterogeneity in small and large ccRCC. A total of 23 small (<4cm) and 24 large (>7cm) ccRCC had 3 regions sampled for evaluation of copy number, clear-cell A/clear-cell B (ccA/ccB) classification, and cell cycle progression (CCP) score. Small tumors have less genomic complexity and significantly fewer subclonal CNV events. Pre-treatment genomic characterization based on a single biopsy in small ccRCC can provide insight into biologic potential to make clinical decisions.

Figure 1. Assessment of the frequency of common CNV driver alterations important in clear cell kidney cancer

a) CNV events analyzed per tumor (n=44) with gains portrayed in red and losses in blue. b) CNV events analyzed between large (n=22) and small tumors (n=22) * $p < 0.05$, # $p < 0.05$ (FDR correction applied) c) Shared CNV events by region (n=41).

Figure 2. Analysis of the frequency and subclonal nature of driver CNV events in small and large clear cell renal tumors

- a) Total CNV events for large and small tumors (all regions considered)(n=44)
- b) Total CNV events for large and small tumors in the TCGA cohort from single region (n=263)
- c) Subclonal CNV events for large and small tumors (n=41)

Figure 3. Transcriptomic classification by the ccA/ccB profile by tumor size in clear cell renal tumors. 100 regions were ccA and 26 regions were ccB. The distribution of ccA/ccB differed between small and large tumors (p=0.024)

Figure 4. Minimal Intratumoral Spearman Correlation of ccA/B and CCP genes by Small and Large Tumor Size (Tumor rank from lowest to highest correlation)

Evaluation of the minimal correlation between regions by size category showed that there was significantly more intratumoral heterogeneity in large tumors $p=0.004$).

Table 1

List of tumor characteristics for small and large clear cell renal tumors in the cohort.

	Small Tumor (≤ 4 cm) (n=23)	Large Tumor(> 7 cm) (n=24)	P Value
Age, median (range), y	60.5 (33–82)	66.5 (49–94)	0.04
Sex			
Male, No. (%)	14 (60.9)	17 (70.8)	0.47
Female, No. (%)	9 (39.1)	7 (29.2)	
Tumor Sizes, median (range), cm	2.5 (1.2–3.9)	9.25 (7–19)	<0.001
Stage, No. (%)			
T1	22 (95.7)	0 (0)	<0.001
T2	0 (0)	5 (20.8)	
T3	1 (4.3)	17 (70.8)	
T4	0 (0)	2 (8.3)	
Tumor Grade, No. (%)			
G1	2 (8.7)	0 (0)	<0.001
G2	20 (87.0)	5 (20.8)	
G3	1 (4.3)	11 (45.8)	
G4	0 (0)	8 (33.3)	
Sarcomatoid component, No. (%)			
Negative	23 (100)	21 (87.5)	0.08
Positive	0	3 (12.5)	
Rhabdoid component, No. (%)			
Negative	23 (100)	21 (87.5)	0.08
Positive	0	3 (12.5)	
MVI, No. (%)			
Negative	23 (100)	19 (79.2)	0.021
Positive	0	5 (20.8)	
Necrosis, No. (%)			
Negative	22 (95.7)	9 (37.5)	<0.001
Positive	1 (4.3)	15 (62.5)	
Regional Lymph Node Metastasis, No. (%)			
Negative	23 (100)	22 (91.7)	0.16
Positive	0	2 (8.3)	
Distant Metastasis, No. (%)			
Negative	23 (100)	19 (79.2)	0.021
Positive	0	5 (20.8)	

Abbreviations: MVI, micro vessel invasion