Expression of hypoxia-inducible factors in clear-cell renal cell carcinoma tumors of adults with and without obstructive sleep apnea

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ABSTRACT

Introduction: Upregulation of hypoxia-inducible factors (HIF) is an important pathologic feature shared by clear-cell renal cell carcinoma (ccRCC) and obstructive sleep apnea (OSA). It is unclear whether OSA alters ccRCC pathogenesis via HIF expression. This study aimed to characterize differences in HIF expression in ccRCC tumors in patients with and without OSA. We hypothesized that a diagnosis of OSA was associated with increased HIF expression.

Methods: A cohort of adults who underwent nephrectomy for ccRCC was identified. OSA diagnosis was determined with preoperative STOP-BANG scores or polysomnography, selecting 20 individuals with and 20 without OSA. Tumor sections were immunohistochemically stained for HIF-1α & HIF-2α and assessed by an expert uropathologist.

Results: The OSA group exhibited a higher prevalence of hypertension (95% vs. 50%, p=0.001) and greater median body mass index (BMI) (34.8 vs. 29.05, p=0.006). Tumor grades were higher in the OSA group (p=0.039). No differences were noted in tumor stages. Samples of ccRCC

KEY MESSAGES

- Obstructive sleep apnea (OSA) and clear-cell renal cell carcinoma (ccRCC) share pathologic expression of hypoxia-inducible factors (HIF).
- The current study examines HIF expression in ccRCC tumors of patients with and without OSA.
- Patients with OSA demonstrated higher levels of HIF-1 positivity and higher tumor grades compared to the non-OSA group.
- No differences were observed in HIF-2 expression.

tumors in the OSA group demonstrated a higher prevalence of HIF-1α positivity (80% vs. 50%, p=0.048), although median histoscores were not different (4 vs. 2.5, *p*=0.260). Neither median HIF-2α histoscores (1 vs. 2, p=0.306) nor expression (histoscore >0; 74% vs. 75%, p=0.927) was statistically significant.
Conclusions: In OSA patients, ccRCC tumors exhibited higher HIF-1α positivity and tumor grades; however, no significant differences in median HIF histoscores, HIF-2α expression, or tumor stage was found. Future studies can use our results to perform formal sample size calculations and elucidate the role of OSA in the pathogenesis of ccRCC.

INTRODUCTION

Clear cell renal cell carcinoma (ccRCC) is the most common type of kidney cancer. In over 80% of the cases, ccRCC arises from mutations in the von Hippel-Lindau (VHL) gene, which result in the stabilization of hypoxia-inducible factors (HIF-1α and HIF-2α).[1-3] Under normal conditions, HIF proteins play a key role in cellular oxygen-sensing pathways, primarily because VHL-dependent degradation or inactivation requires normal oxygen levels.[4,5] Thus, in hypoxic or VHL-null environments (e.g., ccRCC), HIF proteins escape destruction and upregulate a multitude of genes involved in oncogenesis, angiogenesis, and cancer progression.[6] The relevance of the HIF pathway has led to preclinical research for drug targeting HIF proteins in ccRCC.[7,8]

Although the most important prognostic factor for ccRCC is the pathological stage at diagnosis, early detection is limited by the asymptomatic nature of RCC and the lack of evidence-based strategies for routine screening.[9] Consequently, more than half of cases of RCC are detected incidentally.[10,11] Identifying risk factors associated with the development or prognosis of the disease is essential in improving patient outcomes.

One such risk factor is OSA, a sleep disorder characterized by chronic intermittent hypoxia, sleep fragmentation.[12] Patients with OSA display elevated plasma levels of HIF-1α, reflecting the presence of persistent intermittent hypoxic events.[13] OSA and ccRCC also share several risk factors such as obesity, and hypertension.[14] Recent data has suggested a link between OSA and cancer, with one study demonstrating that patients diagnosed with OSA had an elevated overall incidence of cancer.[15] In animal models, it has been shown that both sleep fragmentation as well as intermittent hypoxia accelerate tumor growth and increase tumor invasiveness.[16-18]

Despite these findings, few human studies have been specifically dedicated to how the hypoxic environment induced by OSA may affect the pathogenesis and prognosis of ccRCC. In one study, surgical specimens of kidney cancer patients with OSA showed higher Fuhrman grades than those without it.[19] Overall, identifying a link between OSA and HIF expression in ccRCC is confounded by many variables, including the lack of routine screening for OSA and the prognostic differences of specific HIF subtypes in ccRCC. As such, the relationship between a diagnosis of OSA and the level of HIF expression in ccRCC is still unclear. The present study aimed to characterize the differences in HIF expression in ccRCC tumors of patients with and

without OSA. We hypothesized that a diagnosis of OSA would result in increased HIF expression in these tumors as compared to patients without a history of OSA.

METHODS

Study population

The present is a retrospective, single-center, cohort study performed at Henry Ford Health (Detroit, Michigan, USA) and reported according to the Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) recommendations.[20] A graphical overview of study methodology is represented by Figure 1. After obtaining institutional review board approval (IRB# 16040-01), we identified adults with ccRCC who underwent either partial or total nephrectomy at Henry Ford Health Main Hospital between 2017 to 2022 via an electronic record query. Due to the accessibility of tissue samples, screening was performed in reverse chronological order, starting with the most recent patient. Patients were required to have (a) histologically confirmed clear cell renal cell carcinoma (ccRCC); and (b) a prospectively collected STOP-BANG questionnaire by the pre-operative clinic and/or prior polysomnography (PSG) results. The diagnosis of OSA was defined as a STOP-BANG score of at least 5 and/or a prior PSG-confirmed diagnosis of OSA.[21] Any patient without PSG-confirmed OSA or who did not receive pre-operative STOP-BANG screening was excluded. Screening was performed until a consecutive sample of 20 patients with OSA and 20 patients without OSA was achieved. We are not aware of previous studies that have estimated the prevalence of HIF expression according to OSA diagnosis. Thus, sample size was determined by convenience and availability of funding. As such, this study is considered exploratory in nature.

Data collection & storage

After identification, a REDCap database was established to securely store patient data. Demographic information was collected including age at date of surgery, ethnicity, BMI, legal sex. Past medical history was collected including tobacco use, hypertension, chronic kidney disease (glomerular filtration rate GFR less than 60mL/min), history of lung disease requiring supplemental oxygen, and history of neoadjuvant chemotherapy for ccRCC. Whenever available, the information regarding the diagnosis of OSA was collected including the results of the STOP-BANG questionnaire, any prior PSG, and specific information from the PSG including the apnea hypopnea index (AHI), O2 nadir, and minutes under 88% saturation. The use of any continuous positive airway pressure device was also recorded.

Surgically resected tissue samples were accessed via a request to the tumor bank. Formalin-fixed paraffin-embedded (FFPE) block samples were provided to the Henry Ford Health System Henry Ford Cancer Institute (HFCI) Immunohistochemistry Core for staining procedures.

Immunohistochemical staining

Both HIF-1α and HIF-2α expression were investigated. Immunohistochemistry (IHC) was performed using mouse monoclonal antibodies (AB8366, Abcam) for HIF-1α. For HIF-2α, initial HIF-2α staining was unsuccessful with rabbit polyclonal antibodies (NB-100-122, Novus), so a different antibody was obtained (BL-95-1A2; rabbit recombinant monoclonal; Abcam). Optimal antibody concentrations for staining were empirically determined using human breast cancer, human ovarian cancer, and human bone marrow as the positive human control tissue for

HIF-1α and HIF-2α. High pH retrieval at a dilution of 1:150 with a 30-minute antibody incubation and flex visualization system for used for both HIF-1α and HIF-2α.

Following high pH retrieval, slides were placed in buffer for 5 minutes, then loaded onto the autostainer. They were then rinsed and underwent 5 minutes of endogenous enzyme block (Flex Peroxidase block). Slides were rinsed again. They then underwent a 30-minute antibody incubation, either HIF-1α or HIF-2α. Slides then underwent 5-minute rinse followed by 20 minutes of labeled polymer (FLEX HRP). Slides were again rinsed for 5 minutes. This was followed by 10 minutes of substrate-chromogen (FLEX DAB + Sub-Chromogen), then another rinse. A 5-minute counterstain (FLEX Hematoxylin) was performed. Slides were then rinsed with H2O, then buffer, followed by water before being scanned via whole slide scanning on Aperio CS2 Scanner by Leica Biosystem at 40X magnification.

Assessment of immunohistochemical staining

Cytoplasmic and nuclear HIF expression was scored using the protocol described by Biswas and colleagues.[22] Though we intended to exclusively use nuclear expression, overall HIF nuclear expression was poor. Thus, we opted to combine both cytoplasmic and nuclear staining findings. Intensity of staining was scored as 0 (no staining), 1 (weak), 2 (moderate), and 3 (strong). The percentage of tumor cells stained was scored as 0 (no staining), 1 (1-10%), 2 (11-50%), 3 (51-90%), and 4 (91-100%). A histoscore was then calculated using the product of the intensity and the percentage, giving a resultant score of 0-12. Positive HIF-1α is defined as a histoscore of >2, while positive HIF-2α is defined as any histoscore >0.[22] Scoring was done by an expert uropathologist, who was blinded to the OSA status.

Statistical analysis

All statistical tests were performed using JASP software, version 0.19.0. Ordinal data collected includes STOP-BANG score, tumor grade, tumor stage, and histoscore. Age and BMI are reported as median (range). Categorical data includes hypertensive status. The Mann-Whitney U (Wilcoxon Rank Sum) Test was used to determine statistical significance for ordinal variables. The Mann-Whitney U Test was additionally used for subject age and BMI given that we could not assume normality due to our small sample size.[23] Chi Square statistical testing was used for categorical data. Finally, a z-test for 2 population proportions was used for testing HIF positivity.

RESULTS

Figure 2 displays the study flow chart diagram. In brief, the electronic query identified 231 patients who underwent nephrectomy at our institution. Further screening identified 73 patients with histologically confirmed ccRCC. From this group, 40 patients were identified with confirmed OSA status based off STOP-BANG or chart-documented PSG. No patients in either group had lung disease requiring home supplemental oxygen. Baseline demographic, clinical, surgical, and tumor characteristics are displayed in Table 1.

Of the 20 patients in the OSA group, six had a prior diagnosis of OSA. Five of these patients were diagnosed with PSG at a Henry Ford Sleep Disorder Research Center, and one was diagnosed using a home sleep study. Of the six patients, one had mild OSA, one had moderate OSA, and four were found to have severe OSA. The median AHI was 31.65. The median O2 nadir was 84.5% (69-86%), and the median number of minutes spent under 88% saturation was 18.75 (1-73). Five patients had records reporting the use of CPAP.

Patients with OSA had significantly higher rates of hypertension as compared to patients without OSA (95% vs. 50%, p = 0.001438). Median BMI was significantly higher in the OSA group as compared to the non-OSA group (34.8 vs 29.05, p = 0.00578) (Table 1). STOP-BANG scores were also found to be significantly different (6 vs. 3, p = 0.001). Tumor grades were found to be statistically different between the OSA and non-OSA groups (p = 0.039). Tumor stages, however, were not statistically different (p = 0.644). All statistical results are reported in Table 2.

The staining of HIF-1α was exclusively cytoplasmic. HIF-2α demonstrated both nuclear and cytoplasmic staining, thus scoring was based off combined findings. Representative examples of HIF staining can be visualized in figure 3. The proportion of HIF-1α positive staining (histoscore > 2) was higher in the OSA group (80% vs. 50%, p=0.048). However, median histoscores were not statistically different (4 vs. 2.5, p=0.306). In evaluating HIF-2α, neither median histoscores (1 vs. 2, p = 0.29372) nor positive expression as defined by histoscore > 0 (74% vs. 75%, p = 0.927) were statistically significant. The ccRCC samples of patients with documented use of CPAP showed 80% positivity to HIF-1α and 50% to HIF-2α, with median (range) histoscores of 4 (2-12) and 0.5 (0-9), respectively. Given the small sample size, no statistical comparisons were made with this subgroup.

Regarding normal kidney tissue adjacent to the tumor, we observed that neither HIF-1α & HIF-2α were uniformly expressed in all the normal kidney tissue adjacent to the tumor. Strong cytoplasmic granular expression of HIF-1α was noted in proximal tubules of the cortex and weak granular cytoplasmic staining in distal tubules within the cortex. No staining was seen in medulla, glomeruli, and fibroconnective tissue elements. HIF-2α showed strong granular cytoplasmic staining in distal convoluted tubules of the cortex and weaker cytoplasmic staining in proximal tubules or cortex. No staining was seen within the tissue in the medulla, glomeruli, and fibroconnective tissue. Nerve fibers showed cytoplasmic staining with HIF-2α.

DISCUSSION

The present study explored the proportion and intensity of positive HIF-1α and HIF-2α expression in ccRCC tumors of adults with and without OSA. Overall, we found that ccRCC tumors of patients with OSA had a higher proportion of positive HIF-1α staining and a similar proportion of positive HIF-2α staining as compared to patients without OSA. Regarding staining intensity, this study found no significant differences in the median histoscores for either HIF-1α or HIF-2α.

Our results concerning HIF positivity are in line with previous studies concerning HIF expression patterns in tumors with ccRCC. We found that 55% of tumor specimens demonstrated a HIF-1α histoscore > 3 and 30% of samples showed a HIF-2α histoscore > 2. In comparison, Biswas and colleagues reported high HIF-1α expression in 56% of primary ccRCC tumors and high HIF-2α expression in 40% of tumors.[22] Another study reported that approximately half of ccRCC slides showed low HIF-1α staining (< 25% positive cells stained) while the remaining showed high HIF-1α expression.[24]

Hypoxia in OSA allows HIF proteins to escape VHL-mediated degradation. Indeed, patients with OSA have been shown to have abnormally elevated levels of serum HIF-1α [13]. In ccRCC, mutations in the VHL gene lead to an inappropriate lack of HIF suppression despite normoxia. Thus, both diseases are characterized by pathological HIF expression, acting at different steps in a common VHL/HIF pathway. Stabilized HIF functions as a transcription factor

with many downstream targets, such as VEGF and GLUT1, that regulate angiogenesis, cell proliferation, cell survival, and metabolism of glucose and iron.[6] Upregulation of genes such as these is the proposed mechanism behind certain types of highly angiogenic and glycolytic kidney cancer.[4] Both OSA and ccRCC share the pathologic feature of upregulated HIF-1α expression, whereas studies concerning HIF-2α demonstrate conflicting findings.[22,25,26] For this reason, we hypothesized that patients with concurrent OSA and ccRCC may demonstrate greater dysregulation of HIF as compared to ccRCC patients without OSA.

The correlation between HIF-1α and HIF-2α levels and the degree of tumor invasiveness in ccRCC are complex. Biswas *et al.* characterized ccRCC tumors with variable HIF expression and found that across tumors with high HIF-1α levels, those with concurrent low HIF-2α had a worse prognosis than did those with high HIF-2α.[22] However, they also reported that tumors with high HIF-2α expression demonstrated longer cancer-specific free survival. Other studies have revealed a complex relationship between hypoxia and HIF-2α expression. Cells from rat pheochromocytomas exposed to intermittent hypoxia showed markedly reduced levels of HIF-2α.[26] A lack of HIF-2α has been shown to cause greater oxidative stress in mice with a corresponding reduction in antioxidant enzymes.[25] These studies in conjunction with our own results suggest a need for further exploration of HIF expression in ccRCC on cancer aggression and overall survival.

We saw significant differences in tumor grade, though not in tumor stage, between OSA and non-OSA groups. Interestingly, Vilaseca *et al.* similarly found an association between higher grade and OSA status, and no significant effect on ccRCC tumor stage or size.[19] Multiple studies have demonstrated that patients with a diagnosis of OSA have increased incidence of cancer as well as increased cancer mortality.[27,28] Thus, it is possible that a diagnosis of OSA in ccRCC is correlated with a greater disease burden as seen in tumor grade. The lack of differences in tumor stage can be explained by sample size, center referral for early-stage complex surgeries, and increased rates of incidental detection of ccRCC.

Our study has several strengths, one of which includes our use of a prospectively collected preoperative STOP-BANG score to categorize patients into OSA and non-OSA groups. In a recent meta-analysis, a STOP-BANG >4 in a sleep clinic and surgical population had a positive predictive value of 96% and 79%, respectively, for the diagnosis of OSA, which justified our use of this tool.[21] Given the time and cost burden of PSG, the score allowed us to categorize patients into OSA and non-OSA groups with a standardized questionnaire. Previous studies identified OSA based on pre-existing diagnoses, which can greatly underestimate its prevalence.[19] In fact, most patients with OSA may not be diagnosed at the time of surgery.[29,30] Additionally, we conducted our chart review beginning with the most recently performed nephrectomies. This allowed us to collect the freshest tissue samples, thus reducing the effects of storage time.[31] Moreover, we were able to investigate both HIF-1α and HIF-2α staining and observe differences in the staining patterns of these proteins. Finally, an expert in the field of uropathology independently reviewed and scored tissue samples, providing reliable staining results and interpretation.

We are aware of several limitations in our study. We acknowledge that the STOP-BANG score is a screening and not a diagnostic tool. Future studies could prospectively perform polysomnography on all subjects. Clamping of the kidney for resection will induce ischemia and is expected to alter HIF expression. This is an inherent drawback when analyzing any kidney tumor procured from surgery, but it is expected to affect all tissues similarly. Nonetheless, a

recent study reported that surgical ischemia did not significantly affect HIF expression rates, validating our use of FFPE samples.[32] Additionally, we were not able to focus on nuclear HIF expression due to inadequate staining. Different techniques could be considered to better identify nuclear HIF expression, given the challenges faced during staining. The use of polyclonal antibodies to evaluate HIF-2α is discouraged. Subsequent use of monoclonal antibodies was successful, likely due to the increased specificity of monoclonal antibodies.[33] Future studies may opt to target downstream targets of HIF transcription to better elucidate nuclear HIF expression.

Our study was exploratory in nature with a limited sample size. This prevented sufficient statistical power to perform multivariable analyses, which could have accounted for obvious confounding factors such as sex, obesity, and hypertension, which may be risk factors for OSA and ccRCC. However, our results indicate that concurrent obstructive sleep apnea may alter HIF-1α expression in ccRCC tumors. We also showed a correlation between higher tumor grades and OSA. As survival and disease recurrence were not investigated, the clinical significance of these findings remains to be elucidated. We hope the results obtained from the study will serve as a framework for future, more extensive investigation into the effects of OSA on ccRCC occurrence and recurrence.

CONCLUSIONS

Patients with ccRCC and a concurrent diagnosis of OSA exhibited greater HIF-1α positivity than did patients without OSA. However, differences in median HIF-1α histoscores were not observed, and no differences were seen in HIF-2α positivity or median histoscores. Patients with OSA were observed to have higher tumor grades, though tumor stage did not differ between OSA and non-OSA groups. Further research into disease-free survival and recurrence in patients with ccRCC and OSA is indicated to reveal the clinical significance of our findings.

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FIGURES AND TABLES

Figure 1. Graphical overview of study methodology. A retrospective chart review was performed, identifying 20 patients with obstructive sleep apnea (OSA) and 20 without OSA. Patient tissue samples were then collected and immunohistochemically stained for hypoxia inducible factor (HIF) 1 and HIF-2 and scored by a blinded uropathologist. Image created with *BioRender.com.*

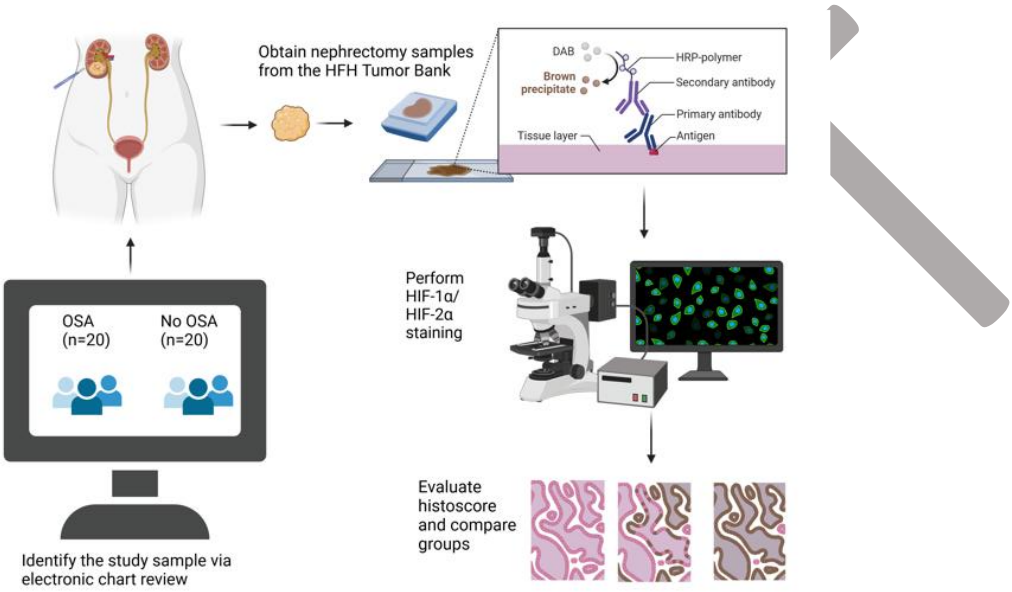

Figure 2. Study selection flowchart. OSA: obstructive sleep apnea.

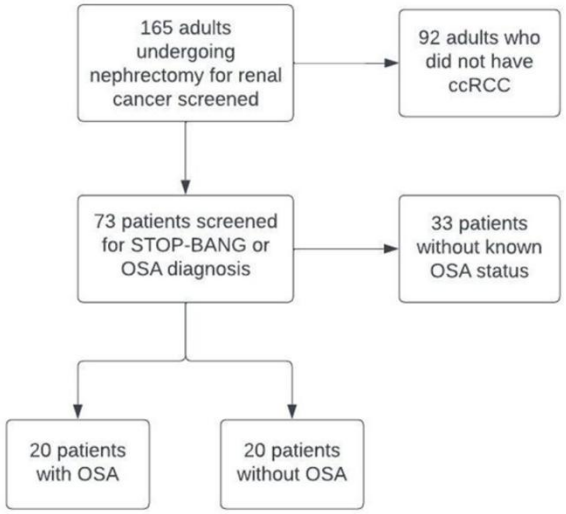

Figure 3. Hypoxia inducible factor (HIF) staining**.** (A) Hematoxylin and eosin (H & E) image of clear cell renal cell carcinoma (ccRCC) (200x magnification). (B) HIF-1 immunohistochemical stain of ccRCC showing nuclear and cytoplasmic reactivity within the tumor cells. (C) H & E image of ccRCC (200x magnification), (D) HIF-2 immunohistochemical stain of ccRCC showing predominantly cytoplasmic reactivity within the tumor cells.

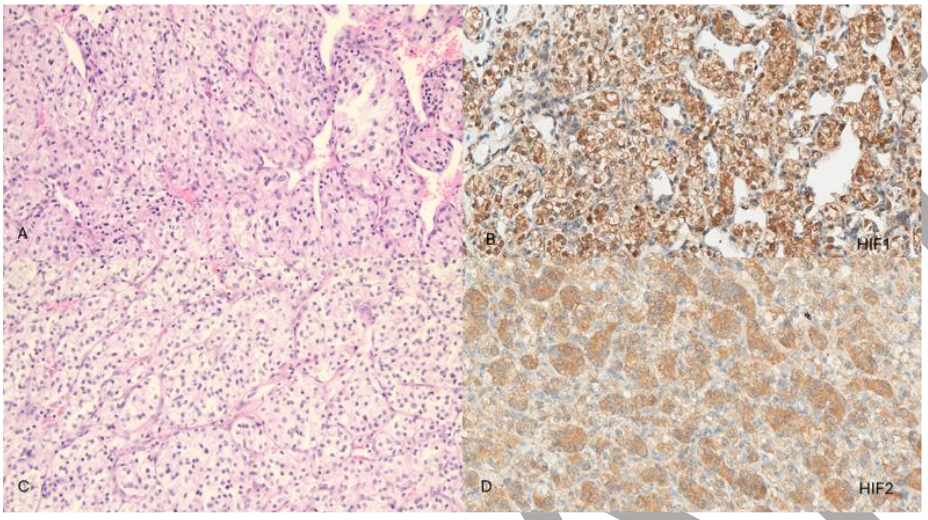

Table 1. Baseline characteristics in the study sample		
Characteristic	**OSA (n=20)**	**Non-OSA (n=20)**
Sex, n (%)		
Men	17 (85%)	13 (65%)
Women	3 (15%)	7 (35%)
Age, median (range)	65.5 (55-80)	61.5 (32-79)
Race, No. (%)		
Asian	2 (10%)	0 (0%)
Black/African American	7 (35%)	4 (20%)
Caucasian	11 (55%)	15 (75%)
Hispanic or Latino	0 (0%)	0 (0%)
Unknown	0 (0%)	1 (5%)
BMI, median	34.8	29.05
Tobacco use		
Current, n (%)	1 (5%)	6 (30%)
Former, n (%)	8 (40%)	14 (70%)
Hypertension, n (%)	19 (95%)	10 (50%)
Chronic kidney disease, n (%)	7 (35%)	5 (25%)
STOP-BANG, median (range)	6 (2–8)	3 (1–4)

Resection type, n (%)		
Radical	14 (70%)	12 (60%)
Partial	6 (30%)	6 (30%)
Total	0 (0%)	2 (10%)
Surgery type, n (%)		
Minimally invasive (robotic)	18 (90%)	15 (75%)
Open	2 (10%)	5 (25%)

OSA: obstructive sleep apnea.

Table 2. Differences in tumor stage, grade, and HIF expression among patients with and without OSA

	OSA (n = 20)	**Non-OSA (n = 20)**	**p**
Tumor stage, n (%)			0.644
pT1a	8 (40%)	8 (40%)	
pT1b	2 (10%)	5 (25%)	
pT2a	0 (0%)	0 (0%)	
pT2b	0 (0%)	0 (0%)	
pT3a	9 (45%)	6 (30%)	
pT3b	1 (5%)	1 (5%)	
Tumor grade, n (%)			0.039
I	0 (%)	0 (%)	
II	4 (20%)	9 (45%)	
III	11 (55%)	10 (50%)	
IV	5 (25%)	1 (5%)	
HIF-1 Histoscore, median (range)	4 (0–12)	2.5 (0–9)	0.260
HIF-2 Histoscore, median (range)	1 (0–9)	2 (0–6)	0.306
HIF-1 positive (%)	80	50	0.048
HIF-2 positive (%)	73.7	75	0.927

HIF: hypoxia inducible factor; OSA: obstructive sleep apnea.