Prognostic Significance of Promoter CpG Island Methylation of Obesity-Related Genes in Patients With Nonmetastatic Renal Cell Carcinoma

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BACKGROUND: Greater than 40% of renal cell carcinoma (RCC) cases in the United States are attributed to excessive body weight. Moreover, obesity also may be linked to RCC prognosis. However, the molecular mechanisms underlying these associations are unclear. In the current study, the authors evaluated the role of promoter methylation in obesity-related genes in RCC tumorigenesis and disease recurrence. METHODS: Paired tumors (TU) and normal adjacent (N-Adj) tissues from 240 newly diagnosed and previously untreated white patients with RCC were examined. For the discovery phase, 63 RCC pairs were analyzed. An additional 177 RCC pairs were evaluated for validation. Pyrosequencing was used to determine CpG methylation in 20 candidate obesity-related genes. An independent data set from The Cancer Genome Atlas also was analyzed for functional validation. The association between methylation and disease recurrence was analyzed using multivariate Cox proportional hazards models and Kaplan-Meier survival analysis. RESULTS: Methylation in neuropeptide Y (NPY), leptin (LEP), and leptin receptor (LEPR) was significantly higher in TU compared with N-Adj tissues (P<0.001) in both the discovery and validation groups. High methylation in LEPR was associated with an increased risk of disease recurrence (hazard ratio, 3.15; 95% confidence interval, 1.23-8.07 (P = .02)). Patients with high methylation in LEPR had a shorter recurrence-free survival compared with patients in the low-methylation group (log-rank, 3 = 2.25 x 10^-3). In addition, high LEPR methylation in TU was associated with more advanced features (P<.05). Consistent with the findings of the current study, lower LEPR expression in TU compared with N-Adj tissues (P = 1.00 x 10^-3) was found in data from The Cancer Genome Atlas. CONCLUSIONS: Somatic alterations of promoter methylation in the NPY, LEP, and LEPR genes are involved in RCC tumorigenesis. Furthermore, LEPR methylation appears to be associated with RCC recurrence. Future research to elucidate the biology underlying this association is warranted. Cancer; 2017;123:3617-27. © 2017 American Cancer Society.

KEYWORDS: kidney cancer, leptin receptor (LEPR), methylation, obesity, recurrence.

INTRODUCTION
Renal cell carcinoma (RCC) accounts for 2% to 3% of all malignancies in adults and comprises 85% of adult kidney cancer cases. Despite improved diagnosis, approximately one-third of patients undergoing nephrectomy progress to metastases or experience local disease recurrence and distant metastases during follow-up. It is important to be able to predict RCC recurrence early and intervene accordingly. Obesity, as measured by body mass index (BMI), influences the development of RCC. Greater than 40% of RCC cases in the United States are associated with excessive body weight. Previous studies have demonstrated an association between overweight and obesity and an increased risk of RCC; however, patients with RCC with a higher BMI are reported to have a significantly better prognosis compared with normal-weight patients, a phenomenon that is well known as the “obesity paradox.” Although strong associations between obesity and RCC were found, to the best of our knowledge only limited studies to date have examined the molecular mechanism linking obesity and RCC tumorigenesis. Epigenetic changes have been suggested as a molecular mechanism mediating this interplay. To date, there are well-established obesity-related genes whose expressions are regulated through epigenetic mechanisms (eg, DNA methylation, histone modification, and microRNAs [miRNAs]). Animal models and human studies clearly have demonstrated methylation changes in promoters of various genes that are implicated in obesity (leptin [LEP], leptin receptor [LEPR], proopiomelanocortin [POMC], melanocortin 4 receptor [MC4R], ubiquitin-associated and SH3 domain-containing A [UBASH3A], and tripartite motif containing 3 [TRIM3]), appetite control and...
metabolism (neuropeptide Y [NPY] and POMC), insulin signaling (insulin-like growth factor 2 [IGF-2] and insulin receptor substrate 1 [IRS-1]), and inflammation (adiponectin [ADIPOQ], ATPase phospholipid transporting 10A [putative] [ATP10A], and tumor necrosis factor [TNF]). However, to the best of our knowledge, the role of DNA methylation in obesity-related genes in the prognosis of RCC has yet to be elucidated.24

Previous studies have shown that aberrant DNA methylations contribute to RCC tumorigenesis13,14 and clinical outcomes.15-18 The methylations of several genes, including DAL-1/4.1B, COL14A1, secreted frizzled related protein 1 (SFRP1), gremlin 1 (GREM1), neuralized E3 ubiquitin protein ligase 1 (NEURL), ladinin 1 (LAD1), and neurofilament heavy (NEFH)22; and DAB2 interacting protein (DAB2IP)23 have been shown to be independent prognostic factors for RCC. For example, van Vlodrop et al22 recently identified 4 methylation markers (GREM1, NEURL, LAD1, and NEFH) that individually predicted the prognosis of patients with clear cell RCC (ccRCC). The 4 markers combined were found to be associated with poorer survival in 2 independent patient series and a third series of cases of ccRCC from The Cancer Genome Atlas (TCGA). To the best of our knowledge, no study to date has reported DNA methylation in obesity-related genes as prognostic markers for patients with RCC.

LEP has been suggested as a biological link between cancer and obesity. LEP exerts its action through LEPR, a class 1 cytokine receptor; disrupted LEPR signaling has been associated with RCC invasion.24-27

In the current study, we sought to investigate the potential role of obesity-related gene methylation in RCC tumorigenesis and recurrence. The methylation of promoter-associated CpG islands of obesity-related genes was assessed by pyrosequencing in RCC tissue pairs of tumor (TU) and normal adjacent tissue (N-Adj) samples and its association with clinicopathologic characteristics and prognosis was evaluated.

MATERIALS AND METHODS
Study Population and Human Tissue Samples
The current study is an ongoing study that has been recruiting patients with RCC from The University of Texas MD Anderson Cancer Center (MDACC) in Houston since 2002. The study design has been described previously.28 Briefly, all recruited cases were patients with newly diagnosed (within 1 year of diagnosis), histologically confirmed, and previously untreated RCC. A total of 240 white patients with RCC were included in the current study. For the discovery population, 63 tissue pairs of TU and N-Adj from the surrounding kidney were collected and for the validation population, 177 tissue pairs were included. The RCC tissues were collected during surgery. The study was approved by the MDACC Institutional Review Board. All participants provided written informed consent before participating in the study.

An independent data set for gene expression including 64 RCC tissue pairs of TU and N-Adj was downloaded from TCGA to provide confirmatory evidence for our methylation findings.29

Epidemiologic and Clinical Data Collection
Epidemiological data were collected by MDACC interviewers during a 45-minute, structured, in-person interview. Data including information regarding history of hypertension (yes/no), smoking status and pack-years of smoking, physical activity and usual weight, and weight at ages 20 years and 40 years were recorded. An individual who had never smoked or had smoked <100 cigarettes in his or her lifetime was defined as a never-smoker. An individual who had smoked at least 100 cigarettes in his or her lifetime but had quit at least 12 months before diagnosis was classified as a former smoker. Current smokers were those who currently were smoking or quit <12 months before diagnosis. The number of pack-years was calculated as the average number of cigarettes smoked per day divided by 20 cigarettes and then multiplied by smoking years. Body mass index (BMI; in kg/m²) was calculated through self-reported usual height and weight. BMI was categorized according to the standard classifications of the World Health Organization (normal [<25 kg/m²], overweight [25-29.9 kg/m²], and obese [≥30 kg/m²]). Participants also reported the average frequency they spent on 5 broad groups of physical activity within the year before the interview. A metabolic equivalent (MET) value was assigned to each activity group and categorized into low (MET <27 per week), medium (MET 27-44.9 per week), and intensive (MET ≥45 per week) activity.30

The clinicopathologic information was abstracted from patient medical records, including pathologic stage of disease, Fuhrman grade, and histology. The pathologic stage was determined according to the 2009 American Joint Committee on Cancer TNM staging system. Tumor cell differentiation was assessed according to the Fuhrman nuclear grade and patients were grouped into low-grade (Fuhrman grades 1 and 2) and high-grade (Fuhrman grades 3 and 4) groups. The tumor histological subtypes were classified according to the 2004 World Health...
Organization classification. All study participants were followed for treatments and disease recurrence. Disease recurrence was defined as local or distant metastatic disease occurring after nephrectomy. The endpoint of the current study was recurrence-free survival (RFS), defined as the time from the date of nephrectomy to the date of disease recurrence or last follow-up.

DNA Extraction and Bisulfite Pyrosequencing
Genomic DNA was extracted using a QIAamp DNAeasy Blood and Tissue Kit (Qiagen, Valencia, Calif) according to the manufacturer’s instructions. The DNA concentration was assessed with an ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, Del). Bisulfite conversion treatment of genomic DNA from each DNA sample was performed using the EZ DNA Methylation Kit (Zymo Research, Orange, Calif), which converts unmethylated cytosines to uracil but leaves methylated cytosines unchanged. We composed a list of obesity-related genes according to a literature search, online database of obesity, and obesity-related pathways and at the end we restricted the gene list to those obesity-related genes whose expression has been reported to be regulated by methylation. The methylation status of the CpG islands in the promoter regions of 20 obesity-related genes in TU and N-Adj samples was analyzed by pyrosequencing at the DNA Methylation Analysis Core at MDACC. Polymerase chain reaction primers were designed using the PyroMark Assay Design 1.0 software (Qiagen, Hilden, Germany) (see Supporting Information Table S1) for the genomic area proximal to the transcription start site of the following genes: ADIPOQ, ADRB3, ATP10A, CREB3L3, CTSZ, FASN, IGF-2, INS, IRS-1, LEP, LEPR, MCAF, NPY, POMC, PPARG, TNF, TRIM3, UCPI, FTO, and UBASH3A. The pyrosequencing was performed using the PSQ HS 96 system (Biotage AB, Uppsala, Sweden) according to the manufacturer’s instructions. Controls for high methylation (SssI-treated DNA), low methylation (DNA amplified by whole-genome amplification), partial methylation (equimolar mixture of SssI-treated and whole gene-amplified DNA), and a blank control without DNA were included in each reaction. The methylation level was calculated using the Pyro-Q CpG 1.0.9v software (Biotage AB). The methylation percentage of each gene was computed as the average of all the assayed CpG sites in the gene.

Statistical Analysis
The chi-square test or Fisher exact test was applied separately to compare the distribution of selected demographic and clinical variables by disease recurrence status. The distribution of each categorical variable was summarized in terms of frequencies and percentages. Differences in continuous variables were evaluated using the Student t test. To determine whether higher methylation was associated with older age and BMI in normal kidney tissues, we measured standardized β-coefficients in normal kidney tissues. A positive estimate (β-coefficient) of the correlation between the 2 variables reflects an increasing methylation response to age and/or BMI factors and a negative estimate reflects a diminishing response to these factors.

Cox proportional hazard models were used to examine the association between obesity-related gene methylation and risk of disease recurrence. Hazard ratios (HRs) and 95% confidence intervals (95% CIs) were estimated. The multivariate regression model was adjusted for age, sex, pathologic stage of disease, grade, smoking status, BMI, hypertension, and histology. RFS curves were determined using Kaplan-Meier analysis and compared with the log-rank test. We also performed independent analyses focusing on the ccRCC histology subtype only. All statistical analyses were conducted using Stata statistical software (version 9.0; StataCorp LLC, College Station, Tex). All tests were 2-sided and a P value of ≤0.05 was considered to indicate statistical significance.

To examine gene expression, we analyzed the data from the TCGA portal. We downloaded level 3 normalized messenger RNA sequencing data of ccRCC from TCGA, and after quality control by removing ineligible samples, the analytic data set consisted of 64 ccRCC tissue pairs of TU and N-Adj samples. The normalized counts were further log2 transformed. The Student t test for paired data was performed to compare the expression levels of selected genes between the samples.

RESULTS

Methylation Levels of Obesity-Related Genes in RCC TU and N-Adj Tissues
The characteristics of the 63 patients with RCC in the discovery phase are shown in Table 1. The mean age of the population was 60 years; the population was largely male and approximately 50% were never-smokers. The majority of the cases were pathologic stage I (65.1%), high-grade (63.5%), and ccRCC (76.2%) (Table 1).

The mean methylation level of the promoter-associated CpG sites in the 20 measured genes in the TU and N-Adj samples are shown in Table 2. For each gene, we defined the mean methylation level of all the CpG sites within the promoter region as this gene’s final DNA
genes for further validation. 

**TABLE 1. Host Characteristics of Patients With RCC in the Discovery and Validation Populations**

| Variable                     | Discovery Set | Validation Set |
|------------------------------|---------------|----------------|
| n = 63                       | n = 177       |
| Demographic variables        |               |                |
| Age (SD), y                  | 60.2 (9.9)    | 59.5 (11.4)    |
| Sex                          |               |                |
| Male                         | 41 (65.1)     | 121 (68.4)     |
| Female                       | 22 (34.9)     | 56 (31.6)      |
| Smoking status               |               |                |
| Never                        | 28 (44.4)     | 91 (51.4)      |
| Former                       | 25 (39.7)     | 61 (34.5)      |
| Current                      | 10 (15.9)     | 25 (14.1)      |
| Physical activity            |               |                |
| D-20                         | 22 (34.9)     | 48 (27.1)      |
| ≥30                          | 12 (19.0)     | 35 (19.8)      |
| Hypertension                 |               |                |
| Yes                          | 34 (54.0)     | 90 (50.8)      |
| No                           | 29 (46.0)     | 87 (49.2)      |
| BMI                          |               |                |
| Normal                       | 21 (33.3)     | 30 (16.9)      |
| Overweight                   | 20 (31.7)     | 51 (28.8)      |
| Obese                        | 20 (31.7)     | 93 (52.5)      |
| Missing data                 | 2 (3.2)       | 3 (1.7)        |
| Pathologic AJCC TNM stage of disease |         |                |
| I                            | 41 (65.1)     | 105 (59.3)     |
| II                           | 6 (9.5)       | 11 (6.2)       |
| III                          | 16 (25.4)     | 61 (34.5)      |
| Fuhrman grade                |               |                |
| Low                          | 22 (34.9)     | 79 (44.6)      |
| High                         | 40 (63.0)     | 97 (54.8)      |
| Missing data                 | 1 (1.6)       | 1 (0.6)        |
| Pathological AJCC TNM stage of disease |         |                |
| I                            | 41 (65.1)     | 105 (59.3)     |
| II                           | 6 (9.5)       | 11 (6.2)       |
| III                          | 16 (25.4)     | 61 (34.5)      |
| Pathologic AJCC TNM stage of disease |         |                |
| I                            | 41 (65.1)     | 105 (59.3)     |
| II                           | 6 (9.5)       | 11 (6.2)       |
| III                          | 16 (25.4)     | 61 (34.5)      |
| Pathologic AJCC TNM stage of disease |         |                |
| I                            | 41 (65.1)     | 105 (59.3)     |
| II                           | 6 (9.5)       | 11 (6.2)       |
| III                          | 16 (25.4)     | 61 (34.5)      |
| Physical activity            |               |                |
| Low                          | 33 (55.0)     | 60 (33.9)      |
| Medium                       | 17 (28.3)     | 34 (19.2)      |
| Intensive                    | 10 (16.7)     | 83 (46.9)      |
| BMI at age 20 y              |               |                |
| Normal                       | 46 (73.0)     | 82 (46.3)      |
| Overweight                   | 10 (15.9)     | 27 (15.3)      |
| Obese                        | 4 (6.4)       | 6 (3.4)        |
| Missing data                 | 3 (4.7)       | 62 (35.0)      |
| BMI at age 40 y              |               |                |
| Normal                       | 23 (36.5)     | 30 (16.9)      |
| Overweight                   | 25 (39.7)     | 50 (28.2)      |
| Obese                        | 11 (17.5)     | 31 (17.5)      |
| Missing data                 | 4 (6.3)       | 66 (37.3)      |
| Histology                    |               |                |
| Clear cell                   | 48 (76.2)     | 143 (80.8)     |
| Other                        | 15 (23.8)     | 34 (19.2)      |
| Disease recurrence           |               |                |
| No                           | 48 (76.2)     | 146 (82.5)     |
| Yes                          | 15 (23.8)     | 31 (17.5)      |
| Dead                         |               |                |
| No                           | 52 (82.5)     | 152 (85.9)     |
| Yes                          | 11 (17.5)     | 25 (14.1)      |

Abbreviations: AJCC, American Joint Committee on Cancer; BMI, body mass index; RCC, renal cell carcinoma; SD, standard deviation.

In addition, we analyzed the effect of demographics and lifestyle factors such as age and BMI on NPY, LEP, and LEPR methylation in normal kidney tissues. We used standardized β-coefficients to measure the estimates of the correlation. In normal kidney tissue, DNA methylation levels of these 3 genes were not found to be significantly associated with BMI. However, there was a significant positive correlation noted between age and LEPR methylation (Rho, 0.26 [P = 3.58E-05]) and between age and LEPR methylation (Rho, 0.43 [P = 4.86E-12]) (see Supporting Information Table S2).

**Methylation Levels of Obesity-Related Genes and Disease Recurrence in Patients With RCC Discovery set**

We evaluated the associations between the methylation of these 3 differentially methylated genes and the risk of disease recurrence. Among the patients, 15 (23.8%) developed disease recurrence. The median follow-up for patients who did not develop disease recurrence was 75.5 months. The distribution of demographic and clinical variables for patients with RCC by disease recurrence status is presented in Table 3.

We used a Cox proportional hazards model adjusted for known and suspected risk factors and confounders to elucidate the association between promoter methylation levels in NPY, LEP, and LEPR and the risk of disease recurrence in patients with RCC. Patients were dichotomized into high-methylation and low-methylation groups according to the median value for each promoter. A multivariate Cox model adjusted for age, sex, pathologic stage of disease, grade, smoking status, BMI, hypertension, and histology identified high methylation levels in LEP as a predictor of disease recurrence in patients with RCC.
## TABLE 2. Methylation and Expression Levels in Patients With RCC in the Discovery and Validation Populations

| Marker | Mean Methylation Level (SD), % | Mean Expression Level (SD) |
|--------|-------------------------------|---------------------------|
|        | Normal                        | Tumor                     |
|        | p                             | q Value                   |
| ADIPOQ | 88.20 (2.13)                  | 87.21 (9.28)              |
| ADRB3  | 10.68 (6.09)                  | 17.38 (10.31)             |
| ATP10A | 3.20 (8.81)                   | 7.01 (9.92)               |
| CREB3L3| 16.11 (4.49)                  | 11.72 (8.31)              |
| CTSZ   | 0.55 (0.43)                   | 1.52 (4.22)               |
| NPY    | 17.68 (12.45)                 | 39.91 (22.07)             |
| FASN   | 2.95 (0.43)                   | 2.98 (0.47)               |
| IGF-2  | 18.40 (4.34)                  | 19.52 (10.38)             |
| INS    | 79.72 (4.31)                  | 71.33 (11.60)             |
| LEP    | 22.62 (6.12)                  | 34.12 (14.42)             |
| IRS-1  | 10.61 (5.98)                  | 16.36 (12.44)             |
| LEPR   | 5.20 (4.13)                   | 16.66 (18.32)             |
| MC4R   | 1.73 (0.90)                   | 2.70 (1.89)               |
| POMC   | 9.18 (1.52)                   | 14.62 (10.55)             |
| PPARG  | 2.15 (6.25)                   | 1.83 (2.34)               |
| TNF    | 39.51 (8.60)                  | 42.48 (19.34)             |
| TRIM3  | 2.96 (2.39)                   | 4.60 (3.88)               |
| UCP1   | 2.77 (0.83)                   | 5.34 (8.07)               |
| FTO    | 1.54 (9.99)                   | 1.54 (9.99)               |
| UBASH3A| 78.99 (6.34)                  | 75.15 (8.96)              |

Abbreviations: ADIPOQ, adiponectin; C1Q and collagen domain containing; ADRB3, adrenoceptor beta 3; ATP10A, ATPase phospholipid transporting 10A (putative); CREB3L3, CAMP responsive element binding protein 3-like 3; CTSZ, cathepsin Z; FASN, fatty acid synthase; FTO, alpha-ketoglutarate-dependent dioxygenase; IGF-2, insulin-like growth factor 2; INS, insulin; IRS-1, insulin receptor substrate 1; LEP, leptin; LEPR, leptin receptor; MC4R, melanocortin 4 receptor; NPY, neuropeptide Y; POMC, proopiomelanocortin; PPARG, peroxisome proliferator activated receptor gamma; RCC, renal cell carcinoma; SD, standard deviation; TCGA, The Cancer Genome Atlas; TNF, tumor necrosis factor; TRIM3, tripartite motif containing 3; UBASH3A, ubiquitin-associated and SH3 domain-containing A; UCP1, uncoupling protein 1.

Bold type indicates the 3 genes studied.

The normalized counts were log2 transformed. The Student t test for paired data was performed to compare the expression levels of selected genes between the tumor and normal adjacent tissue samples.
Subsequently, we evaluated the association between methylation in NPY, LEP, and LEPR and RFS. Kaplan-Meier curves demonstrated a significant association between methylation in LEP and LEPR (low vs high methylation) and RFS (log-rank $P = 2.65E-03$ and $P = .01$, respectively) (Figs. 1B and 1C). No significant differences were found for NPY methylation and RFS ($P = .99$) (Fig. 1A).

### Validation set
We then used an additional 177 RCC tissue pairs to validate our findings. The characteristics of patients with RCC are shown in Table 1. Among the patients, 31 (17.5%) developed disease recurrence. The median follow-up for patients who did not develop disease recurrence was 49.4 months. The distribution of demographic and clinical variables for patients with RCC by disease recurrence status is presented in Table 3.

Among demographic and clinicopathologic variables, higher pathologic stage of disease (HR, 7.77; 95% CI, 2.50-24.10 [$P = 4.00E-04$]) and higher Fuhrman grade (HR, 5.52; 95% CI, 1.20-25.28 [$P = .03$]) were found to be associated with an increased risk of disease recurrence.

In the multivariate Cox proportional hazards model, patients with high methylation levels in LEPR were found to have an increased risk of disease recurrence (HR, 3.15; 95% CI, 1.23-8.07 [$P = .02$]) compared with the low-methylation group among patients with RCC as well as in the subset of patients with ccRCC (HR, 6.00; 95% CI, 1.92-18.82 [$P = 2.00E-03$]) (Table 4).

### TABLE 3. Host characteristics by Disease Recurrence Status in Patients With RCC in the Discovery and Validation Populations

| Variable                  | Discovery Set n = 63 | Validation Set n = 177 |
|---------------------------|----------------------|------------------------|
|                           | Recurrence No. (%)   | Recurrence No. (%)     |
|                           | No Recurrence No. (%)| No Recurrence (%)      |
|                           | $P$                  | $P$                    |
| Mean age (SD), y           |                      |                       |
| 62.27 (11.06)              | 59.50 (9.52)         | .35                    |
| Mean pack-y (SD)           |                      |                       |
| 28.41 (23.05)              | 24.00 (20.90)        | .99                    |
| Mean BMI (SD), kg/m²       |                      |                       |
| 31.47 (7.91)               | 28.08 (6.02)         | .08                    |
| Sex                       |                      |                       |
| Male                      | 13 (86.7)            | 22 (71.0)              |
| Female                    | 2 (13.3)             | 9 (29.0)               |
| Smoking status             |                      |                       |
| Never                     | 4 (26.7)             | 17 (54.8)              |
| Former                    | 9 (60.0)             | 10 (32.3)              |
| Current                   | 2 (13.3)             | 4 (12.9)               |
| Pack-y                    |                      |                       |
| 0-30                      | 5 (33.3)             | 7 (22.6)               |
| ≥30                       | 5 (33.3)             | 7 (22.6)               |
| Missing data              | 5 (33.3)             | 17 (54.8)              |
| Hypertension               |                      |                       |
| Yes                       | 10 (66.7)            | 23 (74.2)              |
| No                        | 5 (33.3)             | 8 (25.8)               |
| BMI                       |                      |                       |
| Normal                    | 2 (13.3)             | 5 (16.1)               |
| Overweight                | 6 (40.0)             | 15 (48.4)              |
| Obese                     | 7 (46.7)             | 10 (32.3)              |
| Missing data              | 0                    | 1 (3.2)                |
| Pathologic AJCC TNM stage of disease |                   |                       |
| I                         | 4 (26.7)             | 5 (16.1)               |
| II                        | 3 (20.0)             | 2 (6.5)                |
| III                       | 8 (53.3)             | 24 (77.4)              |
| Fuhrman grade             |                      |                       |
| Low                       | 1 (6.7)              | 2 (6.5)                |
| High                      | 14 (93.3)            | 29 (93.5)              |
| Missing data              | 0                    | 0                      |
| Histology                 |                      |                       |
| Clear cell                | 13 (86.7)            | 24 (77.4)              |
| Other                     | 2 (13.3)             | 7 (22.6)               |
| Abbreviations: AJCC, American Joint Committee on Cancer; BMI, body mass index; RCC, renal cell carcinoma; SD, standard deviation.

(HR, 5.14; 95% CI, 1.07-24.66 [$P = .04$]) as well as in the subset of patients with ccRCC (HR, 5.96; 95% CI, 1.02-34.76 [$P = .05$]) (Table 4).
TABLE 4. Methylation Levels in Selected Genes and RCC Recurrence Risk

| Marker | Discovery Set | Validation Set |
|--------|---------------|----------------|
|        | Patients With RCC | Patients With RCC |
|        | n = 63         | n = 177         |
|        | Recurrence No. (%) | Recurrence No. (%) |
|        | No. (%)         | HR^a (95% CI)  | P   |
|        | No. (%)         |                |     |
| NPY    | Low 7 (22.58) 17 (19.32) | 1 (referent) | .91 | 17 (80.68) 1 (referent) | .71 |
|        | High 8 (25.00) 14 (15.73) | 0.94 (0.31-2.80) |     | 14 (5.32-2.54) | .34 |
| LEP    | Low 2 (6.25) 11 (12.36) | 1 (referent) | .04 | 14 (22.73) 20 (22.73) | 1.48 (0.66-3.32) | .71 |
|        | High 13 (41.94) 20 (22.73) | 1 (referent) | .04 | 14 (5.32-2.54) | .34 |
|        | Low 3 (9.38) 12 (38.71) | 1 (referent) | .98 | 14 (22.73) 20 (22.73) | 1.48 (0.66-3.32) | .71 |
|        | High 12 (38.71) 23 (25.84) | 1 (referent) | .98 | 14 (22.73) 20 (22.73) | 1.48 (0.66-3.32) | .71 |

Abbreviations: 95% CI, 95% confidence interval; ccRCC, clear cell renal cell carcinoma; HR, hazard ratio; LEP, leptin; LEPR, leptin receptor; NPY, neuropeptide Y; RCC, renal cell carcinoma.

^a The HR was derived using a multivariate regression model adjusted for age, sex, American Joint Committee on Cancer TNM stage of disease, grade, smoking status, body mass index, hypertension, and histology.

Figure 1. Kaplan-Meier estimates of recurrence-free survival (RFS) for patients with renal cell carcinoma (RCC) stratified by methylation levels (low [solid line] vs high [dashed line]). RFS of patients with RCC by neuropeptide Y (NPY), leptin (LEP), and leptin receptor (LEPR) methylation levels are shown in the (A-C) discovery set and (D-F) validation set. MST indicates median event-free survival time (shown in months).
Kaplan-Meier analysis and the log-rank test confirmed the prognostic significance of LEPR in this independent set. Patients with high LEPR methylation in the TU tissue had a shorter RFS compared with patients in the low LEPR methylation group ($P = 2.25E{-03}$) (Fig. 1F). The 5-year RFS rate was estimated to be 67% (95% CI, 53%-78%) for patients with high LEPR methylation compared with 93% (95% CI, 85%-97%) for patients with low LEPR methylation ($P = 5.00E{-04}$). There was no significant association noted between NPY and LEPR methylation and RFS in patients with RCC (log-rank $P = .70$ and $P = .09$, respectively) (Figs. 1D and 1E).

External independent TCGA data set
The methylation of CpG islands in gene promoter regions has been widely studied, and this epigenetic event often is linked to gene silencing and the loss of tumor suppressor functions during tumorigenesis. To provide indirect but confirmatory evidence for our methylation findings, we examined the messenger RNA expression of NPY, LEPR, and LEPR in an external independent data set comprising 64 RCC tissue pairs of TU and N-Adj samples downloaded from the TCGA portal.

We analyzed NPY, LEPR, and LEPR expression in 64 RCC tissue pairs of TU and N-Adj samples from the TCGA portal. We observed significantly lower LEPR expression in TU compared with N-Adj tissue ($P = 1.00E{-03}$) (Table 2), which is consistent with our data indicating higher promoter methylation of LEPR in TU compared with N-Adj tissues. This result suggests that the hypermethylation of the CpG islands in the promoter region of LEPR may be a mechanism for downregulating its expression in RCC tumors.

The data found in the TCGA portal regarding NPY and LEPR expression did not provide confirmatory evidence for our methylation results in RCC tissue pairs because no significant differences were found in NPY and LEPR expression between TU and N-Adj tissues ($P = .81$ and $P = .84$, respectively) (Table 2).

Association Between LEPR Methylation Levels and Clinicopathologic Characteristics in Patients With RCC
To determine whether LEPR promoter methylation level is associated with demographic and clinicopathologic characteristics in patients with RCC, we dichotomized the patients into high-methylation and low-methylation groups according to the same median cutoff point for LEPR promoter described previously and analyzed the association between the LEPR methylation level and host characteristics. We found a significant correlation between high LEPR methylation and high pathologic stage of disease ($P = 1.77E{-04}$), and a borderline significant correlation between LEPR methylation and high Fuhrman grade ($P = .05$) (see Supporting Information Table S3). These data indicate that LEPR methylation is an event present in the pathogenesis of RCC and is associated with poor patient prognosis.

DISCUSSION
The results of the current study demonstrate that methylation in NPY, LEPR, and LEPR promoters is involved in RCC tumorigenesis. In addition, the comparison of methylation data between TU and N-Adj tissues revealed that hypermethylation in these particular obesity-related genes was specific for RCC tumors; in addition to NPY, LEPR and LEPR were demonstrated to be low or unmethylated in normal tissues from the surrounding tissues. Our studies revealed aberrations in DNA methylation that clearly distinguished RCC from normal tissues. Moreover, high methylation in LEPR and the clinicopathologic data indicate that promoter hypermethylation in LEPR methylation might be a late event in kidney tumorigenesis and tumor differentiation.

Promoter hypermethylation in VHL, p16INK4a, p14ARF, APC, GSTP1, MGMT, RASSFLA, RARβ2, E-cadherin, and TIMP-3 have been evaluated in kidney tumors; Dulaimi et al demonstrated that aberrant promoter hypermethylation in tumor suppressor and cancer genes may disrupt critical pathways and thus play an important role in kidney tumorigenesis. Recent high-resolution epigenomic and genomic maps of RCC tumors have reported a significantly increased number of hypermethylated loci in RCC tumors compared with controls; the majority of differentially methylated regions in RCC tumors were localized on enhancer regions of the kidney genome. Although a significant number of hypermethylated loci have been identified in RCC, to our knowledge to date, only a few subsets of CpG island methylation have been clinically characterized and the association between hypermethylation and disease-free survival in patients with RCC has been identified in a small number of genes, but not for obesity-related genes.

To our knowledge, the current study is the first in patients with RCC using paired tumor and normal tissue to evaluate the role of obesity-related gene methylation in RCC tumorigenesis and to associate high methylation in
**LEPR** with the risk of disease recurrence in patients with RCC.

In the current study, a borderline significance was obtained for high methylation levels in **LEPR** in patients with poorly differentiated cancers, which indicates that gene methylation of **LEPR** may be a late event during RCC tumorigenesis. There has been strong evidence suggesting an association between **LEPR** expression and tumor aggressiveness, invasion, metastasis, and clinical outcome.35,36 Furthermore, a recent study reported that **LEPR** demonstrated distinct expression patterns in different histological subtypes of thyroid carcinoma and positive **LEPR** expression was associated with a longer disease-free survival in patients with anaplastic thyroid carcinoma.37 In addition to this evidence and consistent with the findings of the current study, a previous study reported that downregulation of **LEPR** expression increases the risk of metastasis.38 Moreover, low **LEPR** expression was found to be associated with more aggressive tumors.38,39 Biologically, methylation in the promoter-associated CpG sites of thyroid cancer cells has been reported to inhibit cell migration and exhibits an antimetastatic effect through **TIMP-1** (TIMP Metalloproteinase Inhibitor 1) expression, an endogenous inhibitor of matrix metalloproteinase 2 (**MMP-2**). In RCC tumors, increased **MMP-2/9** expression was found to be strongly associated with clinical stage of disease and poor prognosis.41

Upregulation of **TIMP-1** has been reported to inhibit metastasis in patients with hepatocellular carcinoma.42 These data support our hypothesis that **LEPR** may inhibit cell migration. Further research regarding the underlying molecular mechanism of **LEPR** and RCC recurrence is warranted.

Although the findings of the current study suggest an association between epigenetic alterations in obesity-related genes and RCC recurrence, to the best of our knowledge the potential clinical relevance and interaction of **LEPR** methylation with obesity/BMI are not fully understood due to the complexity of metabolic cancer pathways, in particular **NPY**, **LEP**, and **LEPR** signaling. In addition, the relationship between obesity and clinical outcome in patients with RCC remains uncertain. Multiple studies, including a recent meta-analysis, have suggested that having a higher BMI is associated with improved outcomes in patients with RCC.7 However, a recent study did not find extreme obesity to be an independent predictor of worse disease recurrence or survival in a multivariate analysis of patients with RCC who were surgically treated.43

Because epigenetic changes are more tissue-specific and the blood cell methylation profile may not indicate the epigenetic state of the tumor, one of the strengths of the current study is the ability to perform methylation analysis in RCC paired tissue samples, thereby demonstrating the important role of methylation in RCC tumorigenesis and clinical outcome. Another strength of this study is that we performed the methylation analysis using a quantitative evaluation of DNA methylation such as pyrosequencing, which may be more optimal for exploring the clinical significance of a given aberrant promoter methylation because qualitative evaluation may have overvalued low-level methylation, which has less clinical significance. Another advantage was the relatively large number of samples analyzed with the discovery and validation phases. The current study also has limitations, and for prognostic purposes in a clinical setting, epigenetic analysis should be detectable in easily accessible samples such as peripheral blood; because of this, the identification and validation of this marker has to be evaluated in other cell-based samples such as paraffin-embedded tissues or circulating cell-free DNA samples. The current study considered only limited CpG sites for each gene promoter region, and therefore we cannot exclude the possibility that other methylation markers may exist and could exhibit significant associations with RCC tumorigenesis and clinicopathologic characteristics.

The findings of the current study demonstrate that methylation in the **NPY**, **LEP**, and **LEPR** promoters is involved in RCC tumorigenesis. In particular, the results presented herein suggest that the novel methylation marker **LEPR** is an independent factor of disease recurrence in patients with RCC. This prognostic significance may constitute a promising tool with which to improve individualized therapy risk stratification. Further research to elucidate the mechanisms and biology underlying the role
of LEPR methylation and RCC tumorigenesis and disease recurrence are needed.

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**CONFLICT OF INTEREST DISCLOSURES**

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**AUTHOR CONTRIBUTIONS**

Julia Mendoza-Pérez: Conception and design, data acquisition and analysis, and article writing and revision. Jian Gu: Article writing and revision. Luis A. Herrera: Article revision. Nizar M. Tannir: Resource provision. Shanyu Zhang: Statistical analysis. Surena Matin: Resource provision. Jose A. Karam: Resource provision. Christopher G. Wood: Resource provision. Xifeng Wu: Administration and supervision, conception and design, data analysis, funding support, and article revision.

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