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Prognostic significance of promoter CpG island methylation of obesity-related genes in non-metastatic renal cell carcinoma patients

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Abstract

Background—Over 40% of renal cell carcinoma (RCC) cases in the US are attributed to excessive body weight. Moreover, obesity may also be linked to RCC prognosis. However, the molecular mechanism underlying these associations are unclear. In the present study, we evaluated the role of promoter methylation in obesity-related genes in RCC tumorigenesis and recurrence.

Methods—Paired tumors (TU) and normal adjacent (N-Adj) tissues of 240 newly diagnosed and previously untreated Caucasian RCC patients were examined. For the discovery phase, 63 RCC-pairs were analyzed. Additional 177 RCC-pairs were evaluated for validation. Pyrosequencing was used to determine CpG methylation in 20 candidate obesity-related genes. An independent TCGA dataset was also analyzed for functional validation. Association between methylation and recurrence was analyzed using multivariate Cox proportional hazards models and Kaplan-Meier survival analysis.

Results—Methylation in *NPY*, *LEP* and *LEPR* was significantly higher in TU compared with N-Adj tissues ($p<0.0001$) in both discovery and validation groups. High methylation in *LEPR* was associated with increased risk of recurrence (HR=3.15; 95%CI: 1.23–8.07; p=0.02). Patients with high-methylation in *LEPR* had shorter recurrence-free survival than low-methylation group (Log-

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Author contribution:

Julia Mendoza-Pérez: conception and design, data acquisition and analysis, manuscript writing and revision; **Jian Gu**: manuscript writing and revision; **Louis A. Herrera**: manuscript 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, manuscript revision.

Rank p=2.25E-03). Additionally, high *LEPR* methylation in TU was associated with more advanced features (p≤0.05). Consistent with our findings, lower *LEPR* expression in TU compared with N-Adj tissues (p=1.00E-03) was found in TCGA data.

Conclusions—Somatic alterations of promoter methylation in *NPY, LEP* and *LEPR* genes are involved in RCC-tumorigenesis. Furthermore, *LEPR* methylation is associated with RCC recurrence. Future research to elucidate the biology underlying this association is warranted.

Keywords

LEPR; obesity; methylation; recurrence; kidney cancer

INTRODUCTION

Renal cell carcinoma (RCC) accounts for 2–3% of all malignancies in adults and comprises 85% of adult kidney cancer[1]. Despite improved diagnosis, a third of patients undergoing nephrectomy progress to metastasis or experience local recurrence and distant metastasis during follow-up[2]. It is important to be able to predict RCC recurrence early and intervene accordingly[3, 4]. Obesity, measured by body mass index (BMI), influences RCC development. More than 40% of RCC cases in US are indeed associated with excessive body weight[5]. Previous studies have revealed the association of overweight and obesity with increased RCC risk[6]; however, patients with higher BMI had a significantly better RCC prognosis compared with normal weight patients, a phenomenon well known as the “obesity paradox”[7]. Although strong associations between obesity and RCC were found, limited studies have studied the molecular mechanism linking obesity and RCC tumorigenesis[5]. Epigenetic changes have been suggested as a molecular mechanism mediating this interplay[8–11]. To date, there are well-established obesity-related genes whose expressions are regulated through epigenetic mechanisms (e.g., DNA methylation, histone modification and miRNAs). Animal models and human studies have clearly demonstrated methylation changes in promoters of various genes that are implicated in obesity (*LEP*, *LEPR*, *POMC*, *MC4R*, *UBASH3A*, *TRIM3*), appetite control and metabolism (*NPY*, *POMC*), insulin signaling (*IGF-2*, *IRS-1*) and inflammation (*ADIPOQ*, *ATP10A*, *TNF*). However, the role of DNA methylation in obesity-related genes in RCC prognosis has yet to be elucidated[12].

Previous studies have shown that aberrant DNA methylations contribute to RCC tumorigenesis[13, 14] and clinical outcomes[15–18]. The methylations of several genes, including *DAL-1/4.1B*[19], *COL14A1*[20], *SFRP1*[21], *GREM1*, *NEURL*, *LAD1* and *NEFH*[22], and *DAB2IP*[23], have been shown as independent prognostic factors for RCC. For example, van Vlodrop *et al.*[22] recently identified four methylation markers, GREM1, NEURL, LAD1 and NEFH, that individually predicted prognosis of ccRCC patients. The four markers combined were associated with poorer survival in two independent patient series and a third series of ccRCC cases from TCGA. No study has reported DNA methylation in obesity-related genes as prognostic markers for RCC patients.

Leptin (*LEP*) has been suggested as a biological link between cancer and obesity. *LEP* exerts its action through leptin receptor (*LEPR*), a class I cytokine receptor; disrupted *LEP*/*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 were evaluated.

MATERIAL AND METHODS

Study population and human tissue samples

This is an ongoing study that has been recruiting RCC patients from the University of Texas MD Anderson Cancer Center in Houston, Texas, since 2002. The study design was 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 Caucasian RCC patients were included in the present 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 the surgery. The study was approved by the MD Anderson Institutional Review Board. All participants provided written informed consent before participating in the study.

An independent dataset for gene expression including 64 RCC tissue pairs of TU and N-Adj was downloaded from The Cancer Genome Atlas (TCGA) to provide confirmatory evidence for our methylation findings[29].

Epidemiologic and clinical data collection

Epidemiological data were collected by MD Anderson interviewers in a 45-min 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, weight at age 20 and 40 years was 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 were currently 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; kg/m^2) 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/m^2; overweight= 25–29.9 kg/m^2; obese≥30 kg/m^2). Participants also reported the average frequency they spent on five broad groups of physical activities in 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)[30].

The clinicopathologic information was abstracted from patient medical records, including pathologic stage, 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 group in low-grade (Fuhrman grade 1 and 2) and high-grade (Fuhrman grade 3 and 4). The tumor histological subtypes were classified according to the 2004 WHO classification. All study participants were followed on treatments and recurrence. Recurrence was defined as local or distant metastatic disease occurring after nephrectomy. The endpoint of this study was recurrence free survival (RFS), defined as the time from the date of nephrectomy to the date of recurrence or last follow-up.

DNA extraction and bisulfite pyrosequencing

Genomic DNA was extracted using a QIAamp DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. DNA concentration was assessed with an ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). Bisulfite conversion treatment of genomic DNA from each DNA sample was done using the EZ DNA Methylation Kit (Zymo Research, Orange, CA), which converts unmethylated cytosines to uracil but leaves methylated cytosines unchanged. We composed a list of obesity-related genes according to literature search, online database of obesity, obesity-related pathways and at the end we have 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, MD Anderson Cancer Center. PCR primers for the genomic area proximal to the transcription start site of the following genes: *ADIPOQ*, *ADRB3*, *ATP10A*, *CREB3L3*, *CTSZ*, *FASN*, *IGF2*, *INS*, *IRS1*, *LEP*, *LEPR*, *MC4R*, *NPY*, *POMC*, *PPARG*, *TNF*, *TRIM3*, *UCP1*, *FTO* and *UBASH3A* were designed using the PyroMark Assay Design 1.0 software, Qiagen (Hilden, Germany) (Supplementary Table S1). The pyrosequencing was performed using PSQ HS 96 system (Biotage AB, Uppsala, Sweden) following the manufacturer's instruction. Controls for high methylation (SssI-treated DNA), low methylation (WGA-amplified DNA), partial methylation (equimolar mixture of SssI-treated and WGA-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, Uppsala, Sweden). The methylation percentage of each gene was computed as the average of all the assayed CpG sites in the gene.

Statistical analysis

The χ^2 test or Fisher's exact test was applied separately to compare the distribution of selected demographic and clinical variables by 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's t-test. To describe weather higher methylation is associated with higher age and BMI in normal kidney tissues, we measured standardized b-coefficients in normal kidney tissues. A positive estimate (ß-coefficient) of the correlation between the two variables reflects an increasing methylation response to the age and/or BMI factors and a negative estimate reflects a diminishing response to the factors.

Cox proportional hazard models were used to examine the association between obesity-related gene methylation and recurrence risk. Hazard ratios (HRs) and 95% confidence intervals (CIs) were estimated. The multivariate regression model was adjusted by age, gender, pathologic stage, grade, smoking status, BMI, hypertension and histology. RFS curves were determined by the Kaplan-Meier analysis and compared by the Log-Rank test. We also performed independent analyses focusing on clear cell RCC (ccRCC) histology subtype only. All statistical analyses were conducted using STATA version 9.0 (Stata corporation, College Station, TX). All tests were two-sided and a P value of ≤0.05 was considered statistically significant.

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

RESULTS

Methylation Levels of obesity-related genes in RCC tumors and normal-adjacent tissues

The characteristics of the 63 RCC patients in the discovery phase are shown in Table 1. The mean age of the population was 60 years old, largely males, ~50% were never smokers. Most 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 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 methylation value. We set a criteria of statistical significance ($p<0.05$) and a minimum Δ-mean of methylation greater than 10% between TU and N-Adj to select methylated genes for further validation. *NPY, LEP* and *LEPR* were the most significantly hypermethylated genes in TU than in N-Adj tissues. The methylation percentage of *NPY, LEP* and *LEPR* were 39.91±22.07, 34.12±14.42 and 16.66±16.32, respectively, in tumor tissues compared to 17.68±12.45, 22.62±6.12 and 5.20±4.13, respectively, in normal adjacent tissues ($p<0.0001$ for all three genes). We then validated these three genes in a large validation set of TU and paired N-Adj tissues. Again, we observed significantly higher methylation in *NPY* (40.21±22), *LEP* (35.21±14.20) and *LEPR* (14.12±11.89) in TU compared with N-Adj tissues where we observed lower methylation in *NPY* (14.38±6.74), *LEP* (22.58±4.84) and *LEPR* (4.63±2.74) ($p<0.0001$ for all three genes) (Table 2).

Additionally, 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 ß-coefficients to measure the estimates of the correlation. In normal kidney, DNA methylation levels of these three genes were not significantly associated with BMI. However, there was a significant positive correlation between age and *LEP* methylation

(Rho=0.26, p=3.58E-05) and between age and *LEPR* methylation (Rho=0.43, p=4.86E-12) (Supplementary Table S2).

Methylation Levels of obesity-related genes and Recurrence in RCC Patients

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

We used Cox proportional hazard model-adjusted for known and suspected risk factors and confounders to elucidate the association of promoter methylation level in *NPY*, *LEP* and *LEPR* with recurrence risk in RCC patients. Patients were dichotomized into high and low methylation groups according to the median value for each promoter. Multivariate Cox model adjusted by age, gender, pathologic stage, grade, smoking status, BMI, hypertension and histology identified high methylation levels in *LEP* as predictor of recurrence in RCC patients (HR=5.14; 95% CI: 1.07–24.66; p=0.04) as well as in the ccRCC subset (HR=5.96; 95% CI: 1.02–34.76; p=0.05) (Table 4).

Subsequently, we evaluated the association between methylation in *NPY*, *LEP* and *LEPR* and RFS. Kaplan-Meier curves showed a significant association between methylation in *LEP* and *LEPR* (low vs. high methylation) and RFS (Log-Rank p=2.65E-03 and p=0.01, respectively) (Figure 1B and 1C). No significant differences were found for *NPY* methylation and RFS (p=0.99) (Figure 1A).

Validation-Set—Then, we used an additional 177 RCC tissue pairs to validate our findings. The characteristics of RCC patients are displayed in Table 1. Among the patients, 31 (17.5%) patients had recurrence. The median follow-up time for patients who did not recur was 49.4 months. The distribution of demographic and clinical variables for RCC patients by recurrence status is presented in Table 3. Among demographic and clinicopathologic variables, higher pathologic stage (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=0.03) were associated with increased risks of recurrence.

In the multivariate Cox proportional hazards model, patients with high methylation levels in *LEPR* had an increased risk of recurrence (HR=3.15; 95% CI: 1.23–8.07; p=0.02) as compared with low methylation group in RCC patients as well in the ccRCC subset (HR=6.00; 95% CI: 1.92–18.82; p=2.00E-03) (Table 4).

Kaplan-Meier analysis and Log-Rank test confirmed the prognostic significance of *LEPR* in this independent set. Patients with high *LEPR* methylation in the tumor tissue had shorter RFS than low *LEPR* methylation group (p=2.25E-03) (Figure 1F). The 5-year RFS rate was estimated at 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 between *NPY* and *LEP* methylation and RFS in RCC patients (Log-Rank p=0.70 and p=0.09, respectively) (Figure 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 is often linked to gene silencing and loss of tumor suppressor functions during tumorigenesis. To provide indirect but confirmatory evidence for our methylation findings, we examined the mRNA expression of *NPY*, *LEP* and *LEPR* in an external independent dataset consisted of 64 RCC tissue pairs of TU and N-Adj samples downloaded from the TCGA portal.

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

The data found in TCGA portal regarding *NPY* and *LEP* expression did not provide confirmatory evidence for our methylation results in RCC tissue pairs, as no significant differences were found in *NPY* and *LEP* expression between TU and N-Adj tissues (p=0.81 and p=0.84, respectively) (Table 2).

Association of *LEPR* methylation levels and clinicopathologic characteristics in RCC patients

To determine whether *LEPR* promoter methylation level is associated with demographic and clinicopathologic characteristics in RCC patients, we dichotomized the patients into high and low methylation groups according to the same median cut-off point of *LEPR* promoter described previously and analyzed the association between *LEPR* methylation level and host characteristics. We found a significant correlation between high *LEPR* methylation and high pathologic stage (p=1.77E-04) and a borderline significant correlation between *LEPR* methylation and high Fuhrman grade (p=0.05) (Supplementary Table S3). These data indicate that *LEPR* methylation is an event present in pathogenesis of RCC and is associated with patient poor prognosis.

DISCUSSION

Our study demonstrates that methylation in *NPY*, *LEP* and *LEPR* promoters is involved in RCC tumorigenesis. Additionally, the comparison of methylation data between tumor and normal adjacent tissues revealed that hypermethylation in these particular obesity-related genes was specific for renal cell carcinoma tumors; besides *NPY, LEP* and *LEPR* showed 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 indicates 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*, *RASSF1A*, *RARβ2*, *E-Cadherin* and *TIMP3* 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[31]. Recent high-resolution epigenomic and genomic map of RCC tumors have reported a significantly increased number of hypermethylated loci in RCC tumors compared with controls; the majority of DMRs (differentially methylated regions) in RCC were localized on enhancer regions of the kidney genome[32]. Although a great number of hypermethylated loci have been identified in RCC[14], to date, only a few subset of CpG island methylation has been clinically characterized and the association of hypermethylation and disease free survival in RCC has been identify in a small number of genes[15, 17, 19, 33, 34], but not for obesity-related genes.

To our knowledge, this is the first study in 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 risk of recurrence in RCC patients.

In our investigation, a borderline significance was obtained for high methylation levels in *LEPR* in 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* displayed distinct expression patterns in different histological subtypes of thyroid carcinoma and positive *LEPR* expression was associated with longer disease-free survival in anaplastic thyroid carcinoma patients[37]. In addition to this evidence and consistent with our findings, a previous study reported that down-regulation of *LEPR* expression increases the risk of metastasis[38]. Moreover, low *LEPR* was associated with more aggressive tumors[38, 39]. Biologically, methylation in the promoter-associated CpG sites of *LEPR* presumably down-regulates *LEPR* expression in RCC tumors, and this assumption is supported indirectly by the information reported in TCGA portal regarding *LEPR* expression in tissue pairs of RCC tumor and normal-adjacent tissues. Epigenetic regulation of *LEPR* expression has been previously suggested in thyroid cancer cells[40]. Nevertheless, direct experimental data showing promoter hypermethylation leading to reduced *LEPR* expression in RCC cells is warranted to provide biological insights into the role of *LEPR* methylation in RCC prognosis.

RCC patients with high methylation in *LEPR* have a higher risk of recurrence and shorter RFS time. Regarding *LEPR* function and biology, we speculated that *LEPR* produced by cancer cells is able to inhibit cell migration and exhibit anti-metastatic effect through *STAT3* (Signal Transducer and Activator of Transcription 3) activation which consequently augments *TIMP1* (TIMP Metallopeptidase Inhibitor 1) expression, an endogenous inhibitor of *MMP2* (Matrix Metalloproteinase 2). In RCC tumors, increased MMP2/9 expression was strongly associated with clinical stage and poor prognosis[41]. Up-regulation of TIMP-1 has been reported to inhibit metastasis in 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.

Even though our findings suggest an association between epigenetic alteration in obesity-related genes and RCC recurrence, the potential clinical relevance and the interaction of

LEPR methylation with obesity/BMI are not fully understood due to the complexity of metabolic cancer pathways in particular leptin/leptin receptor signaling. Additionally, the relationship between obesity and RCC clinical outcome remains uncertain. Multiple studies, including a recent meta-analysis, have suggested that having a higher BMI is associated with improved outcomes in RCC[7]. However, a recent study did not find extreme obesity as an independent predictor of worse recurrence or survival in a multivariate analysis of surgically treated RCC patients[43]

Since epigenetics changes are more tissue-specific and the blood cell methylation profile may not reveal the epigenetic state of the tumor, one of the strengths of this study is the possibility of performed the methylation analysis in RCC paired tissue samples, enlightening the important role of methylation in RCC tumorigenesis and clinical outcome. Another strength of this study is that we performed the methylation analysis through 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 discovery and validation phases. The present study has also limitations and for prognostic purpose 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 present study only considered limited CpG sites for each gene promoter regions, we cannot exclude the possibility that other methylation marks may exist and could exhibit significant associations with RCC tumorigenesis and clinicopathologic characteristics.

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

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

1. Purdue MP, Moore LE, Merino MJ, et al. An investigation of risk factors for renal cell carcinoma by histologic subtype in two case-control studies. Int J Cancer. 2013; 132:2640–2647. [PubMed: 23150424]

2. Escudier B, Porta C, Schmidinger M, et al. Renal cell carcinoma: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. Ann Oncol. 2014; 25:iii49–56. [PubMed: 25210086]
3. Sun M, Shariat SF, Cheng C, et al. Prognostic factors and predictive models in renal cell carcinoma: a contemporary review. Eur Urol. 2011; 60:644–661. [PubMed: 21741163]
4. Eichelberg C, Junker K, Ljungberg B, Moch H. Diagnostic and prognostic molecular markers for renal cell carcinoma: a critical appraisal of the current state of research and clinical applicability. Eur Urol. 2009; 55:851–863. [PubMed: 19155123]
5. McGuire BB, Fitzpatrick JM. BMI and the risk of renal cell carcinoma. Curr Opin Urol. 2011; 21:356–361. [PubMed: 21730854]
6. Renehan AG, Tyson M, Egger M, Heller RF, Zwahlen M. Body-mass index and incidence of cancer: a systematic review and meta-analysis of prospective observational studies. Lancet. 2008; 371:569–578. [PubMed: 18280327]
7. Choi Y, Park B, Jeong BC, et al. Body mass index and survival in patients with renal cell carcinoma: a clinical-based cohort and meta-analysis. Int J Cancer. 2013; 132:625–634. [PubMed: 22610826]
8. Bouchard L, Rabasa-Lhoret R, Faraj M, et al. Differential epigenomic and transcriptomic responses in subcutaneous adipose tissue between low and high responders to caloric restriction. Am J Clin Nutr. 2010; 91:309–320. [PubMed: 19939982]
9. Campion J, Milagro FI, Goyenechea E, Martinez JA. TNF-alpha promoter methylation as a predictive biomarker for weight-loss response. Obesity (Silver Spring). 2009; 17:1293–1297. [PubMed: 19584886]
10. Cordero P, Campion J, Milagro FI, et al. Leptin and TNF-alpha promoter methylation levels measured by MSP could predict the response to a low-calorie diet. J Physiol Biochem. 2011; 67:463–470. [PubMed: 21465273]
11. Milagro FI, Campión J, Cordero P, et al. A dual epigenomic approach for the search of obesity biomarkers: DNA methylation in relation to diet-induced weight loss. FASEB J. 2011; 25:1378–1389. [PubMed: 21209057]
12. Lopomo A, Burgio E, Migliore L. Epigenetics of Obesity. Prog Mol Biol Transl Sci. 2016; 140:151–84. [PubMed: 27288829]
13. Ellinger J, Holl D, Nuhn P, et al. DNA hypermethylation in papillary renal cell carcinoma. BJU Int. 2011; 107:664–669. [PubMed: 20553257]
14. Ricketts CJ, Hill VK, Linehan WM. Tumor-specific hypermethylation of epigenetic biomarkers, including SFRP1, predicts for poorer survival in patients from the TCGA Kidney Renal Clear Cell Carcinoma (KIRC) project. PloS One. 2014; 9:e85621. [PubMed: 24454902]
15. Eggers H, Steffens S, Grosshennig A, et al. Prognostic and diagnostic relevance of hypermethylated in cancer 1 (HIC1) CpG island methylation in renal cell carcinoma. Int J Oncol. 2012; 40:1650–1658. [PubMed: 22327210]
16. Patrício P, Ramalho-Carvalho J, Costa-Pinheiro P, et al. Deregulation of PAX2 expression in renal cell tumours: mechanisms and potential use in differential diagnosis. J Cell Mol Med. 2013; 17:1048–1058. [PubMed: 23890189]
17. Xiao W, Wang J, Li H, et al. Fibulin-1 is down-regulated through promoter hypermethylation and suppresses renal cell carcinoma progression. J Urol. 2013; 190:291–301. [PubMed: 23391467]
18. He W, Li X, Xu S, et al. Aberrant methylation and loss of CADM2 tumor suppressor expression is associated with human renal cell carcinoma tumor progression. Biochem Biophys Res Commun. 2013; 435:526–532. [PubMed: 23643812]
19. Yamada D, Kikuchi S, Williams YN, et al. Promoter hypermethylation of the potential tumor suppressor DAL-1/4.1B gene in renal clear cell carcinoma. Int J Cancer. 2006; 118:916–923. [PubMed: 16152585]
20. Morris MR, Ricketts C, Gentle D, et al. Identification of candidate tumour suppressor genes frequently methylated in renal cell carcinoma. Oncogene. 2010; 29:2104–2117. [PubMed: 20154727]
21. Atschekzei F, Hennenlotter J, Janisch S, et al. SFRP1 CpG island methylation locus is associated with renal cell cancer susceptibility and disease recurrence. Epigenetics. 2012; 7:447–457. [PubMed: 22419128]

22. van Vlodrop IJ, Joosten SC, de Meyer T, et al. A four-gene promoter methylation marker panel consisting of GREM1, NEURL, LAD1 and NEFH predicts survival of clear cell renal cell cancer patients. Clin Cancer Res. 2016 Oct 18.
23. Wang ZR, Wei JH, Zhou JC, et al. Validation of DAB2IP methylation and its relative significance in predicting outcome in renal cell carcinoma. Oncotarget. 2016; 7:31508–19. [PubMed: 27129174]
24. Horiguchi A, Sumitomo M, Asakuma J, et al. Leptin promotes invasiveness of murine renal cancer cells via extracellular signal-regulated kinases and rho dependent pathway. J Urol. 2006; 176:1636–1641. [PubMed: 16952706]
25. Drabkin HA, Gemmill RM. Obesity, cholesterol, and clear-cell renal cell carcinoma (RCC). Adv Cancer Res. 2010; 107:39–56. [PubMed: 20399960]
26. Horiguchi A, Sumitomo M, Asakuma J, et al. Increased serum leptin levels and over expression of leptin receptors are associated with the invasion and progression of renal cell carcinoma. J Urol. 2006; 176:1631–1635. [PubMed: 16952705]
27. Spyridopoulos TN, Petridou ET, Dessypris N, et al. Inverse association of leptin levels with renal cell carcinoma: results from a case-control study. Hormones. 2009; 8:39–46. [PubMed: 19278051]
28. Clague J, Lin J, Cassidy A, et al. Family history and risk of renal cell carcinoma: results from a case-control study and systematic meta-analysis. Cancer Epidemiol Biomarkers Prev. 2009; 18:801–807. [PubMed: 19240244]
29. The Cancer Genome Atlas. TCGA portal overview. https://tcga-data.nci.nih.gov/tcga/. Accessed January, 2016.
30. Ainsworth BE, Haskell WL, Whitt MC, et al. Compendium of physical activities: an update of activity codes and MET intensities. Med Sci Sports Exerc. 2000; 32:S498–504. [PubMed: 10993420]
31. Dulaimi E, Ibanez de Caceres I, Uzzo RG, et al. Promoter hypermethylation profile of kidney cancer. Clin Cancer Res. 2004; 10:3972–3979. [PubMed: 15217927]
32. Hu CY, Mohtat D, Yu Y, et al. Kidney cancer is characterized by aberrant methylation of tissue specific enhancers that are prognostic for overall survival. Clin Cancer Res. 2014; 20:4349–60. [PubMed: 24916699]
33. Ahmad ST, Arjumand W, Seth A, Saini AK, Sultana S. Methylation of the APAF-1 and DAPK-1 promoter region correlates with progression of renal cell carcinoma in North Indian population. Tumour Biol. 2012; 33:395–402. [PubMed: 21922274]
34. Peters I, Gebauer K, Dubrowinskaja N, et al. GATA5 CpG island hypermethylation is an independent predictor for poor clinical outcome in renal cell carcinoma. Oncol Rep. 2014; 31:1523–1530. [PubMed: 24549248]
35. Zhang Y, Liu L, Li C, Ai H. Correlation analysis between the expressions of leptin and its receptor (ObR) and clinicopathology in endometrial cancer. Cancer Biomark. 2014; 14:353–359. [PubMed: 25171477]
36. Milosevic VS, Vukmirovic FC, Krstic MS, Zindovic MM, Lj Stojanovic D, Jancic SA. Involvement of leptin receptors expression in proliferation and neoangiogenesis in colorectal carcinoma. J BUON. 2015; 20:100–108. [PubMed: 25778303]
37. Fan YL, Li XQ. Expression of leptin and its receptor in thyroid carcinoma: distinctive prognostic significance in different subtypes. Clin Endocrinol. 2015; 83:261–267.
38. Rodrigues PR, Maia LL, Santos M, et al. Leptin receptor expression and Gln223Arg polymorphism as prognostic markers in oral and oropharyngeal cancer. Genet Mol Res. 2015; 14:14979–14988. [PubMed: 26634459]
39. Menghi F, Orzan FN, Eoli M, et al. DNA microarray analysis identifies CKS2 and LEPR as potential markers of meningioma recurrence. Oncologist. 2011; 16:1440–1450. [PubMed: 21948653]
40. Cheng SP, Liu CL, Hsu YC, Chang YC, Huang SY, Lee JJ. Regulation of leptin receptor expression in human papillary thyroid cancer cells. Biomed Pharmacother. 2012; 66:469–473. [PubMed: 22560341]

41. Kugler A, Hemmerlein B, Thelen P, Kallerhoff M, Radzun HJ, Ringert RH. Expression of metalloproteinase 2 and 9 and their inhibitors in renal cell carcinoma. J Urol. 1998; 160:1914–1918. [PubMed: 9783985]

42. Wang N, Zhu M, Tsao SW, Man K, Zhang Z, Feng Y. Up-regulation of TIMP-1 by genipin inhibits MMP-2 activities and suppresses the metastatic potential of human hepatocellular carcinoma. PloS One. 2012; 7:e46318. [PubMed: 23029478]

43. Blute ML, Zorn K, Grimes M, et al. Extreme Obesity Does Not Predict Poor Cancer Outcomes Following Surgery for Renal Cell Cancer. BJU Int. 2016; 118:399–407. [PubMed: 26589741]

Précis

In this study of 240 tumor-normal pairs from renal cell carcinoma (RCC) patients, we found methylation in obesity-related genes was involved in RCC tumorigenesis and prognosis. Specifically, high methylation in leptin receptor (*LEPR*) gene was associated with more advanced tumor features and correlated with short recurrence-free survival.

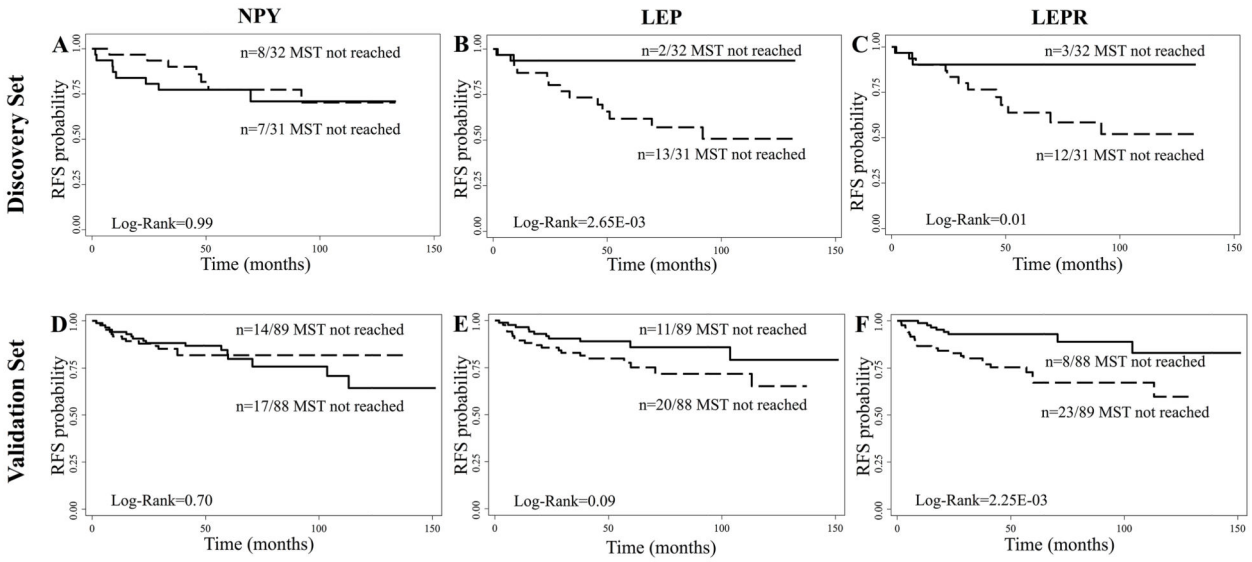

Figure 1.

Kaplan-Meier estimates of recurrence free survival (RFS) for RCC patients stratified by methylation levels (Low-solid line vs High-dashed line). RFS of RCC patients by *NPY*, *LEP* and *LEPR* methylation levels in (A–C) discovery set and (D–F) validation set. MST indicates median event-free survival times (in months).

Table 1

Host Characteristics of RCC patients in the Discovery and Validation Populations

	Discovery set (n=63)	***Validation set (n=177)***
Variable	**n(%)**	**n(%)**
Age Mean (SD)	60.2(9.9)	59.5(11.4)
Pack-years Mean (SD)	25.3(21.3)	28.1(29.0)
BMI Mean (SD)	28.9(6.6)	31.0(6.6)
Gender		
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)
Pack-years		
0~30	22(34.9)	48(27.1)
≥30	12(19.0)	35(19.8)
Missing	29(46.0)	94(53.1)
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	2(3.2)	3(1.7)
Pathologic Stage		
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(63.5)	97(54.8)
Missing	1(1.6)	1(0.6)
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		
Normal	46(73.0)	82(46.3)
Overweight	10(15.9)	27(15.3)
Obese	4(6.4)	6(3.4)

	Discovery set (n=63)	***Validation set (n=177)***
Variable	**n(%)**	**n(%)**
Missing	3(4.7)	62(35.0)
BMI at age 40		
Normal	23(36.5)	30(16.9)
Overweight	25(39.7)	50(28.2)
Obese	11(17.5)	31(17.5)
Missing	4(6.3)	66(37.3)
Histology		
Clear Cell	48(76.2)	143(80.8)
Other	15(23.8)	34(19.2)
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)

SD: standard deviation, BMI: body mass index

Table 2

Methylation and expression levels in RCC patients in the Discovery and Validation Populations

	Discovery set (n=63)				*Validation set (n=177)*			*Functional Validation TCGA-set (n=64)*		
	Mean Methylation Level, % (SD)				**Mean Methylation Level, % (SD)**			**Mean Expression Level, (SD)***		
Marker	**Normal**	**Tumor**	**p value**	**q value**	**Normal**	**Tumor**	**p value**	**Normal**	**Tumor**	**p value**
ADIPOQ	88.20(2.13)	87.21(9.28)	0.41	0.27						
ADRB3	10.68(5.08)	17.38(10.31)	1.94E-05	4.45E-05						
ATP10A	3.20(3.81)	7.01(9.92)	1.70E-03	2.17E-03						
CREB3L3	16.11(4.45)	11.72(8.31)	5.29E-04	7.58E-04						
CTSZ	0.55(0.43)	1.52(4.22)	0.08	0.07						
NPY	**17.68(12.45)**	**39.91(22.07)**	**1.33E-08**	**1.52E-07**	**14.38(6.74)**	**40.21(22.00)**	**5.682E-35**	0.11(1.61)	−0.32(1.17)	0.81
FASN	2.95(0.49)	2.98(0.47)	0.66	0.40						
IGF2	18.40(4.34)	19.52(10.38)	0.42	0.27						
INS	79.72(4.31)	71.33(11.60)	9.45E-07	2.71E-06						
LEP	**22.62(6.12)**	**34.12(14.42)**	**4.11E-08**	**2.36E-07**	**22.58(4.84)**	**35.21(14.20)**	**1.796E-22**	0.94(2.44)	0.85(1.76)	0.84
IRS1	10.61(5.98)	16.36(12.44)	1.93E-03	2.21E-03						
LEPR	**5.20(4.13)**	**16.66(16.32)**	**5.61E-07**	**2.14E-06**	**4.63(2.74)**	**14.12(11.89)**	**1.073E-20**	**9.68(0.7)**	**9.23(0.87)**	**1.00E-03**
MC4R	1.73(0.90)	2.70(1.89)	2.12E-04	3.47E-04						
POMC	9.18(1.52)	14.62(10.55)	1.29E-04	2.46E-04						
PPARG	2.15(5.25)	1.83(2.34)	0.41	0.27						
TNF	39.51(8.60)	42.48(19.34)	0.23	0.18						
TRIM3	2.86(2.35)	4.60(3.88)	2.86E-03	2.98E-03						
UCP1	2.77(0.80)	5.34(8.07)	0.01	0.01						
FTO	1.54(0.98)	1.54(0.86)	0.97	0.56						
UBASH3A	78.99(6.26)	75.15(8.96)	4.05E-03	3.87E-03						

*The normalized counts were log2 transformed. Paired t-test was performed to compare the expression levels of selected genes between the tumor and normal samples

Table 3

Host characteristics by Recurrence Status in RCC patients in the Discovery and Validation Populations

	Discovery set (n=63)			*Validation set (n=177)*		
Variable	**Recurrence n(%)**	**No Recurrence n(%)**	**p value**	**Recurrence n(%)**	**No Recurrence n(%)**	**p value**
Age Mean (SD)	62.27(11.06)	59.50(9.52)	0.35	63.61(7.92)	58.62(11.83)	0.03
Pack-years Mean (SD)	28.41(23.05)	24.00(20.90)	0.59	42.67(44.66)	25.18(24.20)	0.04
BMI Mean (SD)	31.47(7.91)	28.08(6.02)	0.08	28.88(4.35)	31.46(6.95)	0.05
Gender						
Male	13(86.7)	28(58.3)		22(71.0)	99(67.8)	
Female	2(13.3)	20(41.7)	0.04	9(29.0)	47(32.2)	0.73
Smoking Status						
Never	4(26.7)	24(50.0)		17(54.8)	74(50.7)	
Former	9(60.0)	16(33.3)		10(32.3)	51(34.9)	
Current	2(13.3)	8(16.7)	0.17	4(12.9)	21(14.4)	0.91
Pack-years						
0~30	5(33.3)	17(35.4)		7(22.6)	41(28.1)	
≥30	5(33.3)	7(14.6)		7(22.6)	28(19.2)	
Missing	5(33.3)	24(50.0)	0.25	17(54.8)	77(52.7)	0.52
Hypertension						
Yes	10(66.7)	24(50.0)		23(74.2)	67(45.9)	
No	5(33.3)	24(50.0)	0.26	8(25.8)	79(54.1)	4.20E-03
BMI						
Normal	2(13.3)	19(39.6)		5(16.1)	25(17.1)	
Overweight	6(40.0)	14(29.2)		15(48.4)	36(24.7)	
Obese	7(46.7)	13(27.1)		10(32.3)	83(56.8)	
Missing	0	2(4.2)	0.13	1(3.2)	2(1.4)	0.02
Pathologic Stage						
I	4(26.7)	37(77.1)		5(16.1)	100(68.5)	
II	3(20.0)	3(6.3)		2(6.5)	9(6.2)	
III	8(53.3)	8(16.7)	1.68E-03	24(77.4)	37(25.3)	1.16E-07

	Discovery set (n=63)			*Validation set (n=177)*		
Variable	**Recurrence n(%)**	**No Recurrence n(%)**	**p value**	**Recurrence n(%)**	**No Recurrence n(%)**	**p value**
Fuhrman grade						
Low	1(6.7)	21(43.8)		2(6.5)	77(53.1)	
High	14(93.3)	26(54.2)		29(93.5)	68(46.9)	
Missing	0	1(2.1)	7.38E-03	0	1(0.7)	2.14E-06
Histology						
Clear Cell	13(86.7)	35(72.9)	0.28	24(77.4)	119(81.5)	0.60
Other	2(13.3)	13(27.1)		7(22.6)	27(18.5)	

SD: standard deviation, BMI: Body mass index

Table 4

Methylation levels in Selected Genes and RCC recurrence Risk

	Discovery Set				*Validation Set*			
	RCC patients (*n=63*)				**RCC patients (*n=177*)**			
Marker	**Recurrence n(%)**	**No Recurrence n(%)**	**HR*(95%CI)**	**p value**	**Recurrence n(%)**	**No Recurrence n(%)**	**HR*(95%CI)**	**p value**
NPY								
Low	7(22.58)	24(77.42)	1(ref)		17(19.32)	71(80.68)	1(ref)	
High	8(25.00)	24(75.00)	0.94(0.31–2.80)	0.91	14(15.73)	75(84.27)	1.16(0.53–2.54)	0.71
LEP								
Low	2(6.25)	30(93.75)	1(ref)		11(12.36)	78(87.64)	1(ref)	
High	13(41.94)	18(58.06)	5.14(1.07–24.66)	0.04	20(22.73)	68(77.27)	1.48(0.66–3.32)	0.34
LEPR								
Low	3(9.38)	29(90.63)	1(ref)		8(9.09)	80(90.91)	1(ref)	
High	12(38.71)	19(61.29)	1.02(0.14–7.71)	0.98	23(25.84)	66(74.16)	3.15(1.23–8.07)	0.02
	ccRCC patients (*n=48*)				**ccRCC patients (*n=143*)**			
NPY								
Low	7(29.17)	17(70.83)	1(ref)		14(19.72)	57(80.28)	1(ref)	
High	6(25.00)	18(75.00)	1.70(0.39–7.40)	0.48	10(13.89)	62(86.11)	1.05(0.42–2.62)	0.91
LEP								
Low	2(8.33)	22(91.67)	1(ref)		9(12.50)	63(87.50)	1(ref)	
High	11(45.83)	13(54.17)	5.96(1.02–34.76)	0.05	15(21.13)	56(78.87)	1.41(0.58–3.42)	0.44
LEPR								
Low	2(8.33)	22(91.67)	1(ref)		6(8.33)	66(91.67)	1(ref)	
High	11(45.83)	13(54.17)	1.59(0.22–11.59)	0.65	18(25.35)	53(74.65)	6.00(1.92–18.82)	2.00E-03

HR* Multivariate regression model adjusted by age, gender, stage, grade, smoking status, BMI, hypertension and histology