Somatic expression of ENRAGE is associated with obesity status among patients with clear cell renal cell carcinoma

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An association between obesity and development of clear cell renal cell carcinoma (ccRCC) has been established in the literature; however, there are limited data regarding the molecular mechanisms that underlie this association. Therefore, we used a multistage design to identify and validate genes that are associated with obesity-related ccRCC. We conducted a microarray study and compared gene expression between obese and non-obese subjects in ccRCC tumors and patient-matched normal kidney tissues. Analyses were stratified by smoking status and subsequently performed on the combined cohort. The primary objective was to identify genes where the fold change of ccRCC tumor expression between obese and non-obese subjects was different than the fold change in the patient-matched normal kidney tissue. Thus, we utilized a mixed model and evaluated the tissue type-by-obesity status interaction term. Targeted validation was performed using reverse transcription–polymerase chain reaction (RT–PCR) on an independent cohort. ENRAGE was identified in the microarray study and subsequently validated using RT–PCR to have a statistically significant tissue type-by-obesity status interaction. Specifically, although ENRAGE is similarly expressed across obese and non-obese subjects in normal tissue, it is upregulated in the patient-matched ccRCC tumor tissue. Additionally, ENRAGE is upregulated in tumors that are wild-type for the von Hippel Lindau gene and in tumors for subjects with poorer overall survival. In summary, we provide evidence that overexpression of ENRAGE in ccRCC tumor tissue is an obesity-associated somatic alteration. Upregulation of ENRAGE could lead to local, autocrine stimulation of the RAGE receptor and thus support cancer progression.

Introduction

The incidence of renal cell carcinoma (RCC) in the USA continues to rise and this trend cannot be fully attributed to incidental detection via increased use of abdominal imaging. Related to this, obesity rates have been rising steadily in the USA for several decades and obesity incidence highlight a need to deepen our understanding of this well-known association. Although the role of obesity in RCC development is well documented, the specific molecular alterations within RCC tumors that underlie this association remain largely unknown. To date, the few studies that have investigated the molecular associations between obesity and RCC have all used a candidate gene approach focusing exclusively on germline evaluation of genes that had been shown previously to be associated with obesity. Specifically, a germline single-nucleotide polymorphism in the fat mass and obesity-associated (FTO) gene has been shown to also be associated with an increased risk of young age onset of RCC (4). Additionally, germline single-nucleotide polymorphisms in the angiotensinogen (AGT) gene have been shown to be associated with RCC risk among hypertensive or overweight subjects (5). And most recently, Shu et al. (6) demonstrated that germline single-nucleotide polymorphisms in the mammalian target of rapamycin pathway are associated with energy-related risk factors and RCC risk. These studies all investigated the association of host (germline) genetics and the origin of RCC for obesity-related genes. However, somatic alterations also play an important role in carcinogenesis and thus it is important to additionally study associations between obesity and subtypes of RCC defined by the presence or absence of specific somatic alterations.

Motivated by this, our hypothesis is that obesity causes specific somatic alterations in kidney cells that ultimately lead to gene expression changes in RCC tumors. Thus, genes that have differential expression in RCC tissues from obese and non-obese patients can be used to define RCC molecular subtypes. This molecular phenotyping can then be used to better understand the underlying biology of obesity-related RCC risk, as has been done in other cancers (7). As such, herein, we employed a genome-wide approach to provide the first evidence of RNA molecular alterations within RCC tissues that are linked to obesity. Specifically, we used the Affymetrix U133 Plus 2.0 platform to compare genome-wide gene expression patterns from clear cell RCC (ccRCC) tumors and patient-matched normal tissue between obese and non-obese subjects. From these analyses, we identified that ccRCC tumors from obese subjects overexpress the gene ENRAGE when compared with ccRCC from non-obese subjects; however, we did not find a detectable difference in ENRAGE expression from normal kidney tissue samples between obese and non-obese subjects. This suggests that ENRAGE is a somatic molecular alteration that is associated with obesity-related ccRCC. We then validated this finding using reverse transcription–polymerase chain reaction (RT–PCR) on an independent set of patient-matched ccRCC and normal kidney samples from obese and non-obese patients. Furthermore, we report for the first time that ENRAGE expression is higher in ccRCC tumors that are wild-type for the von Hippel Lindau (VHL) gene (mutated in ~50–60% of all ccRCC) and in tumors for ccRCC subjects with poorer overall survival.

Materials and methods

Ethics statement

This study was approved by the Mayo Clinic Institutional Review Board. All participants provided written consent to participate in this study.

Overview

For this investigation, we employed a multistage design that allowed us to stratify by smoking status, the other main risk factor for RCC. Briefly, in stage 1, we used the Affymetrix platform to compare gene expression levels between obese and non-obese ccRCC patients in both tumor tissue and patient-matched normal kidney tissue. The goal of stage 1 was to identify candidate genes that are differentially expressed between obese and non-obese patients at different levels in ccRCC tissues and normal kidney tissues. Then, in stage 2, we
applied the same approach; however, this time we included only patients with a history of smoking. We acknowledged that false positives are inherent in whole genome discovery-based inquiries. Thus, this second step allowed us to filter our candidate list of genes from stage 1 to only those genes where an association with obesity was strong enough to be detected among subjects with the other major epidemiological risk factor for ccRCC. Subsequently, we combined the data from stage 1 and stage 2 and performed an analysis on the combined data to further focus our list of candidates. With the final list of candidates in hand, we then performed RT–PCR validation on our top gene candidates (stage 3) using an independent set of patient-matched ccRCC and normal kidney tissues. We provide more detail on the design and selection of the patients for each stage in the sections below.

**Patient selection**

**Stage 1:** affymetrix microarrays on ccRCC subjects who self-reported to be a non-smoker. For this first stage, we sought to remove possible confounding variables that might occur from exposure to smoking, the other primary risk factor for ccRCC. To do so, we selected only ccRCC subjects who self-reported no history of smoking and compared gene expression between obese and non-obese subjects across their ccRCC tumors as well as their normal kidney samples. Upon approval from the Mayo Clinic Institutional Review Board, we identified subjects treated with radical nephrectomy or nephron-sparing surgery for unilateral, sporadic ccRCC between 2000 and 2007 from our ongoing Mayo Clinic Nephrectomy Registry. We then excluded all subjects who self-reported a history of smoking as well as subjects with late-stage tumors (pT4) and those with high-grade tumors (grade 4). The decision to remove smokers was based on the fact that smoking represents the only other widely accepted epidemiological risk factor for ccRCC development and thus we wanted to match by smoking status. Based on these criteria, we identified 42 subjects who self-reported to be a non-smoker and that had fresh-frozen normal kidney and tumor tissue available for study; 12 of which were obese and 30 that were not obese. We defined obese as anyone who had a body mass index (BMI) >30 at time of surgery and non-obese as anyone who had BMI <30.

**Stage 2:** affymetrix microarrays on ccRCC subjects who self-reported to be a smoker. As noted above, because cigarette smoking is the other widely acceptable risk factor, we performed a two-stage design stratifying by smoking status. Thus, we repeated our design and analysis from stage 1 but this time we only used subjects who reported >20 pack-years of smoking on either the questionnaire or during the patient history at time of surgery. Other than being a smoker, the subjects in stage 2 were similar to stage 1 (i.e. unilateral, sporadic, pT stages 1–3, grades 1–3). As such, stage 2 consisted of 23 ccRCC subjects that self-reported >20 pack-years of smoking and that had both fresh-frozen normal kidney and tumor tissue available for study; 7 of which were obese and 16 that were non-obese. We used the same criteria to define obesity status as described above for stage 1.

**Stage 3:** RT–PCR validation on ccRCC subjects who self-reported to be a non-smoker. With our discovery-based steps complete, the objective of stage 3 was to seek independent validation of the candidate genes we identified in stages 1 and 2. The subjects in stage 3 consisted of 73 patients that self-reported no history of smoking and that had both fresh-frozen normal kidney and tumor tissue available for study; 19 of which were obese and 54 that were not obese. In the validation cohort, we defined obese as BMI >35 at time of surgery and non-obese as BMI <30.

**Tissue preparation and laboratory assays**

**Samples for genomic profiling.** An experienced urologic pathologist identified fresh-frozen blocks with representative tumor and normal tissue blocks for each patient involved in stages 1–3. Subsequently, an experienced histotechnologist macrodissected 5 μ sections from representative fresh-frozen tissue blocks for each tumor and normal tissue block. The Mayo Biospecimen Accessioning and Processing Core performed RNA extractions using kits and protocols from the Qiagen miRNEasy kit and Qiagen Qiacube instrument. The DNA was DNase treated on the column prior to elution. The RNA quantity was assessed by Nanodrop Spectrophotometer and integrity of the RNA by Agilent.

**Affymetrix microarrays.** Microarray analysis was conducted according to the manufacturer’s instructions for the Affymetrix One Cycle Target Labeling and Control Reagents kit (Affymetrix, Santa Clara, CA). Briefly, complementary DNA (cDNA) was generated from 5 μg of total RNA using SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA) and T7 Oligo(dT) primer. Subsequently, the products were column purified (Affymetrix) and then in vitro transcribed to generate biotin-labeled complementary RNA. The IVT products were then column purified, fragmented and hybridized onto Affymetrix U133 Plus 2.0 GeneChips® at 45 °C for 16h. Subsequent to hybridization, the arrays were washed and stained with streptavidin–phycoerythrin, then scanned in an Affymetrix GeneChip® Scanner 3000 (Affymetrix). All control parameters were confirmed to be within normal ranges before normalization was initiated. The data discussed in this publication have been deposited in NCBI’s Gene Expression Omnibus and are accessible through GEO Series accession number GSE46699 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE46699).

**Microarray data normalization and statistical methods.** The data used herein are comprised of two batches of samples that were processed at two different time periods (Supplementary Methods, available at Carcinogenesis Online). Base-2 logarithm-transformed intensity data from the two batches of samples were normalized within each batch using frozen robust multi-array analysis (8). Frozen robust multi-array analysis was specifically designed to pre-process arrays in batches and subsequently allow the data to be combined for downstream analyses.

We provide a schematic of the samples used in stage 1 and stage 2 in Figure 1. Linear mixed models were fit to the normalized intensity data for each probe set in order to identify genes that have somatic alterations that are associated with obesity-related ccRCC. Within the linear mixed model, tissue type (tumor/normal), obesity status (obese/non-obese) and an obesity status-by-tissue type interaction were included as fixed effects, whereas a random intercept was fit on a per subject basis to account for the patient-matched tumor–normal specimens. The obesity status-by-tissue type interaction was included to specifically test if the fold change of expression for obese versus

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**Fig. 1.** Experimental design for stage 1 and stage 2. Four normal tissues did not have adequate RNA for microarray analysis.
non-obese subjects in the tumor tissues is different than the fold change in the patient-matched normal kidney tissues. That is, we were not seeking to identify genes that had an overall obesity effect. Instead, we aimed to identify somatic molecular alterations that are associated with obesity-related ccRCC. Subsequently, to help interpret the interaction term, we calculated fold change of normal kidney expression in obese subjects relative to non-obese subjects as well as fold change of ccRCC tumor expression in obese relative to non-obese subjects. We report the corresponding fold change P values, which test the null hypothesis that the fold change equals a value of one.

Stage 1 and stage 2 data were analyzed separately and then subsequently combined and analyzed as a whole. Because false positives are inherent in whole genome discovery-based studies, both the stage 2 analysis and the analysis of the combined stage 1 and stage 2 data were used to facilitate removal of false positives. Probesets with an obesity status-by-tissue type interaction P value <0.01 in stage 1 were identified as having a potential association with obesity-specific somatic alterations in ccRCC and therefore were further evaluated in stage 2. Due to the small sample size available in stage 2, a liberal obesity status-by-tissue type interaction P value <0.05 was applied at this stage. Subsequently, any genes that passed stage 1 and stage 2 were required to maintain an interaction P value <0.01 in the analysis of the combined stage 1 and stage 2 data. We acknowledge that the corresponding P value thresholds do not account for multiple testing at the conservative Bonferroni level; however, probesets that were consistently identified in stage 1, stage 2 and subsequently in the analysis of the combined stage 1 and stage 2 data were deemed to be worthwhile candidates for further validation in stage 3 acknowledging that some genes might be false positives. Statistical tests were performed using SAS 9.3 or a Linux release of R version 2.14. All probeset-to-gene mapping was done using the hu133plus2.db (version 2.9.0).

**Gene expression by Fluidigm quantitative PCR.** Samples were reverse transcribed according to the manufacturer’s instructions for the High Capacity Reverse Transcription Kit (Applied Biosystems, Foster City, CA). Briefly, 50 ng of total RNA was reverse transcribed in a 20 μl reaction mixture containing 0.1 μl 100 mM dNTP, 2.0 μl RT buffer, 1.0 μl of reverse transcriptase (50 U/μl), 2 μl of RT primer. The reaction mixture was mixed and incubated as follows: 25°C for 10 min, 37°C for 2 h and then 85°C for 5 min, followed by a 4°C hold. Preamplification of cDNA was initiated by creating a pool of 24 TaqMan messenger RNA (mRNA) assays at a final concentration of 0.2X for each assay. The reaction was then performed in a 5 μl reaction mixture containing 2.5 μl TaqMan PreAmp Master Mix (2X), 1.25 μl of 24-pooled TaqMan assay mix (0.2X) and 1.25 μl of cDNA. The preamplification PCR was performed according to the following cycling conditions: one cycle 95°C for 10 min, 14 cycles at 95°C for 15 s and then 60°C for 4 min. After preamplification PCR, the product was diluted 1:5 with dH2O and stored at −20°C until needed for amplification. Quantitative PCR of the mRNA targets was carried out using the 48.48 dynamic array (Fluidigm, South San Francisco, CA) following the manufacturer’s protocol. Briefly, a 5 μl sample mixture was prepared for each sample containing 2x TaqMan Universal Master Mix (with UNG), 20X GE Sample Loading Reagent and each of diluted preamplified cDNA. Five microliters of assay mix was prepared with one 20X TaqMan mRNA assay (final concentration 1X) and 2X Assay Loading Reagent. The dynamic array was primed with control line fluid in the integrated fluidic circuit (IFC; Fluidigm) controller and samples and assay mixes were loaded into the appropriate inlets. The chip was then returned to the IFC controller for loading and mixing, and then placed in the BioMark Instrument for PCR at 50°C for 2 min and 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. The data were analyzed with the Real-Time PCR Analysis Software (Fluidigm).

**RT–PCR data normalization and statistical methods.** Normalization was carried out as discussed previously (9). In brief, the negative CT (denoted hereafter as −CT) values for the two control genes (POLR2A and ACTB) were averaged on a per sample basis and the average was subtracted from the −CT value for each sample. As was done for the Affymetrix microarray data, linear mixed models were fit to the normalized −CT data for each gene in order to determine if somatic gene alterations are associated with obesity-related ccRCC. Within the linear mixed model, tissue type (tumor/normal), obesity status (obese/non-obese) and an obesity status-by-tissue type interaction were included as fixed effects, whereas a random intercept was fit on a per patient basis to account for the patient-matched tumor–normal samples. Genes with an obesity status-by-tissue type interaction P value <0.05 were deemed to be statistically significant.

Statistical methods for testing association of ENRAGE with VHL and overall survival

Clinical data, level 3 gene level RNAseq data and VHL mutation status were retrieved from the The Cancer Genome Atlas (TCGA) data portal for 446 ccRCC (KIRC) subjects. Using the TCGA data, Welch’s t-statistic was used to test for differential ENRAGE expression between mutant and wild-type VHL. The association of ENRAGE expression with overall survival was done separately using the combined stage 1 and stage 2 microarray data and also using the TCGA KIRC data. Cox proportional hazards regression was used to evaluate the association between ENRAGE expression and overall survival after adjusting for age; hazard rates and P values are reported. In the Cox model, ENRAGE expression was dichotomized as low versus high using the 75th percentile of normalized ENRAGE expression. The 75th percentile was calculated separately for the microarray and KIRC RNA sequencing data. A P value threshold of 0.05 was used for all analyses.

**Results**

**Patient characteristics**

We provide a comparison of demographic and clinical characteristics between obese and non-obese patients for each of the three stages in Table I. We observed no meaningful differences in age categories or gender between obese and non-obese subjects across the three stages. In all three stages, non-obese patients tended to have higher grade tumors than obese subjects. In stage 2, non-obese subjects were more likely to have higher pathologic tumor stage compared with obese subjects. However, the pathologic tumor stage distribution was more similar between obese and non-obese subjects in design stages 1 and 3.

**Discovery of genes associated with obesity-related ccRCC (stage 1 and stage 2)**

We identified three Affymetrix probesets that passed our criteria for validation and these probesets mapped to one non-coding RNA and two genes: NCRNA00263, ENRAGE and EFCAB6 (Supplementary Table 1, available at Carcinogenesis Online). To note, we were specifically interested in identifying somatic molecular alterations that were associated with obesity-related ccRCC. That is, we aimed to identify genes where the fold change of tumor expression in obese relative to non-obese subjects was different than the fold change in the patient-matched normal kidney tissue. In doing so, we observed no detectable difference in expression for either NCRNA00263 or EFCAB6 between obese and non-obese subjects in normal kidney tissue; however, both tended to be downregulated in obese in comparison with non-obese subjects in the corresponding patient-matched ccRCC tumor tissue. Conversely, we observed no detectable difference in expression for ENRAGE between obese and non-obese subjects in normal kidney tissue; however, ENRAGE was significantly upregulated in obese in comparison with non-obese subjects in the corresponding patient-matched tumor tissue. Thus, ENRAGE and EFCAB6 were selected for follow-up validation in stage 3. Validation of the non-coding RNA NCRNA00263 is work in progress.

**Independent RT–PCR validation (stage 3)**

Although both EFCAB6 and ENRAGE demonstrated similar effect sizes in the RT–PCR analyses as was observed in stages 1 and 2 (Supplementary Table 2, available at Carcinogenesis Online), only ENRAGE had a statistically significant obesity status-by-tissue type interaction in stage 3 (Table II) implying that the fold change of expression between obese and non-obese subjects is different in normal kidney tissue in comparison with the ccRCC tumor tissue. Specifically, in stage 1, ENRAGE had an obesity status-by-tissue type interaction P value of 0.0083; the fold change of expression between obese and non-obese subjects was 1.0044 (P = 0.95) in normal kidney tissue and 1.24 (P = 0.0036) in the patient-matched tumor tissue. That is, there was no observed difference in ENRAGE expression relative to obese and non-obese subjects in normal kidney tissue. However, in the patient-matched tumor tissue, ENRAGE expression was upregulated in obese subjects relative to non-obese subjects. These results were further confirmed in stage 2 with an interaction P value of 0.030; the fold change of expression between obese and non-obese subjects was 1.02 (P = 0.88) in normal kidney tissue and 1.53 (P = 0.0049) in the patient-matched tumor tissue. Performing quantitative RT–PCR on an independent cohort of 73 subjects with no history of smoking (stage 3), we further confirmed these results with an interaction
Somatic expression of ENRAGE in ccRCC

Although obesity is currently believed to be associated with the development of several human cancers, a positive association with the development of RCC specifically is well reported in the literature. Moreover, investigators have also reported evidence of a dose–response relationship with more obese subjects at greater risk (10). Based on the current literature, there is strong evidence regarding the role of obesity in the etiology of RCC; however, what remains unclear is exactly how obesity acts within the body to increase a person’s risk of developing RCC. Related to this, potential mechanisms linking obesity and RCC have been suggested. Perhaps, the most familiar mechanism involves an increase in cancer risk due to an increase in the circulating blood levels of free estrogens (11,12). In addition to elevated estrogen levels, obese individuals also have increased serum levels of insulin-like growth factor I, a particularly potent cell mitogen ing obesity and RCC have been suggested. Related to this, potential mechanisms linking obesity and RCC risk include elevated insulin levels of insulin-like growth factor I, a particularly potent cell mitogen.

Association of ENRAGE with VHL

VHL mutations are prevalent in ccRCC and an association between VHL and obesity in ccRCC has been suggested in the literature (2). Thus, we evaluated the association between VHL and ENRAGE tumor expression using the TCGA ccRCC data and observed that ENRAGE gene expression is upregulated 1.54-fold in VHL wild-type tissue and 1.48 (P = 0.091) in the patient-matched tumor tissue.

Association of ENRAGE with overall survival

Using only the tumor samples from our combined stage 1 and stage 2 microarray data, we evaluated the association between ENRAGE expression in ccRCC tumors and overall survival. After adjusting for age, we observed that ccRCC subjects with high ENRAGE tumor expression have worse outcomes than subjects with low ENRAGE expression (hazard rate = 5.60, P = 0.013). Although the effect size was attenuated, these results were further confirmed using the TCGA ccRCC RNA sequencing data (hazard rate = 1.49, P = 0.027).

Discussion

Although obesity is currently believed to be associated with the development of several human cancers, a positive association with obesity and RCC has been suggested. Related to this, potential mechanisms linking obesity and RCC risk include elevated insulin and increased insulin insensitivity, greater sympathetic activity or hypertension, elevated cholesterol levels, immune system dysfunction, lower levels of vitamin D, diets higher in overall calories but lower in antioxidants, less physical activity and genetic predisposition (host genetics) (14). Although several proposed theories exist as to why obesity increases the risk of RCC, it remains unclear what specific molecular mechanism(s) link obesity with RCC development.

Herein, we employed Affymetrix gene microarray technology followed by independent RT–PCR validation to report that the obese versus non-obese fold change for the ENRAGE mRNA transcript (also known as S100A12) is significantly different in ccRCC tumor tissues in comparison with the patient-matched normal kidney tissues. Specifically, ENRAGE is upregulated in ccRCC tissues of obese subjects compared to non-obese subjects.

Table I. Demographic and clinicopathologic characteristics for subjects in each stage of the multistage design

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<th>Table I. Demographic and clinicopathologic characteristics for subjects in each stage of the multistage design</th>
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<tr>
<td>Affymetrix microarray</td>
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<td>Stage 1: all non-smokers</td>
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<tr>
<td>Non-obese, 30 (71%)</td>
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Table II. Results for stage 1, stage 2, combined samples from stage 1 and stage 2, and stage 3

Obese versus non-obese fold changes

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<th>Table II. Results for stage 1, stage 2, combined samples from stage 1 and stage 2, and stage 3</th>
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<td>Fold change in tumor tissue (P value)</td>
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<td>Stage 1</td>
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<td>Stage 2</td>
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<td>Combined (stages 1 + 2)</td>
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<td>Stage 3 (RT–PCR)</td>
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Fold change of expression in obese relative to non-obese patients and interaction P values are provided.

P value of 0.025; the fold change of ENRAGE expression comparing obese and non-obese subjects was 1.03 (P = 0.91) in normal kidney tissue and 1.48 (P = 0.091) in the patient-matched tumor tissue.
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Compared with non-obese subjects; however, ENRAGE expression is similar in normal kidney tissues from obese and non-obese subjects. As such, we provide the first empirical evidence of a specific somatic alteration within ccRCC tumors that is associated with obesity in humans. This observation is compelling given that obesity has been postulated to potentiate cancer development through excessive formation of altered glycosylation endproducts that exert pro-growth effects via binding to the RAGE receptor (15). Related to this, ENRAGE is a primary ligand for the RAGE receptor and therefore upregulation of ENRAGE could lead to local, autocrine stimulation of the RAGE receptor and thus support cancer progression. Indeed, upregulation of ENRAGE in RCC tumors appears to be a viable means through which obesity could increase the risk of RCC. Additionally, S100 proteins are involved in cell cycle progression and differentiation and have been shown to be useful biomarkers of inflammation (16,17). Furthermore, Yamaoka et al. (18) recently performed a genome-wide microarray experiment followed up by RT–PCR validation and demonstrated that ENRAGE has a positive association with visceral fat adiposity. Lastly, we additionally showed that ENRAGE expression is upregulated in VHL wild-type tumors and that subjects with high ENRAGE tumor expression have worse overall survival. These results further support that somatic expression of ENRAGE defines a unique molecular and clinical phenotype of ccRCC and helps to underscore our suggestion that ENRAGE may also define a risk factor-specific alteration in RCC as well. That being said, we acknowledge that our findings must be validated at the protein level and there is a need to link alterations in ENRAGE to obesity-related ccRCC in a more robust epidemiologic study design. Particularly, using a large case–control or prospective cohort study where associations with obesity are specifically explored with molecularly defined ccRCC subtypes, included ENRAGE expression. Such a study would also allow adjustment for additional risk factors for ccRCC and obesity (e.g. smoking, hypertension, diabetes, etc.), evaluation of evidence for a dose–response relationship, and largely, exploration of interactions with age, race and gender.

In the results discussed herein, we acknowledge that only 130 probesets passed stage 1 at a P value threshold of 0.01, which is considerably fewer than what would be expected by chance. When reviewing the data more carefully, it was determined that a robust standard error should be used in calculating the test statistics due to non-constant variance and thus all analyses were rerun using the empirical option in PROC MIXED (SAS v9.3) and these results are provided in Supplementary Table 3, available at Carcinogenesis Online. Unfortunately, this reanalysis was performed after the RT–PCR study was completed and thus after genes were chosen for validation. However, as shown in Supplementary Table 3, available at Carcinogenesis Online, the two genes (ENRAGE and EFCAB6) and one non-coding RNA (NCRNA00263) that were originally identified were also identified in the reanalysis of the data. When analyzing whole genome microarray data, Barton et al. (19) recently discussed that misspecification of statistical models should be investigated in instances where there are fewer significant P values than would be expected by chance and the authors discussed an example where this was due to heteroskedasticity. They observed that misspecification mostly resulted in false negatives, which is what we observed as well. Many of the additional genes provided in Supplementary Table 3, available at Carcinogenesis Online, have differential expression between obese and non-obese subjects in the normal kidney tissue and not in the patient-matched ccRCC tumor tissue, including two probesets targeting insulin-like growth factor 1. Conversely, there are genes that have differential expression in the tumor tissue and not in the normal kidney tissue, including RBPMS which has been shown to be associated with VHL in RCC (20). As such, there are additional interesting targets that should be further evaluated.

We used a discovery-based approach to identify obesity-specific somatic molecular alterations associated with ccRCC development. The key limitations of our approach include the focus on expression changes at the RNA level (compared with DNA or protein expression), the overall limited generalizability (tertiary referral center, >95% of patients are Caucasian) and our inability to adjust for other factors that are known to be highly correlated with ccRCC and obesity (e.g. smoking, hypertension, hyperlipidemia, etc.). We also acknowledge that the cohorts we analyzed had differences in pathology in the ccRCC tumors that were studied between obese and non-obese patients. Particularly, obese patients in all three stages of the design tended to have lower grade disease. This finding was not surprising as we have shown previously that obesity is linked to lower grade tumors and better outcomes in ccRCC (21). Unfortunately, we were left with a limited number of patients that had fresh-frozen tissue available after matching on smoking status, the other widely acceptable risk factor, and thus were not able to additionally match on pathology. As a result, we acknowledge that our results could imply that ENRAGE is more highly expressed in low- versus high-grade tumors. However, this is highly unlikely considering that we also showed that increased ENRAGE tumor expression is associated with worse prognosis. Furthermore, the recently discovered association of visceral fat adiposity and ENRAGE (18) provides additional evidence that it is more likely that ENRAGE is associated with obesity and not tumor grade. However, as discussed above, our results need to be validated in a larger and more robust study design. Specific strengths of our design include the use of only ccRCC subtype (the most common histologic subtype), exclusion of late-stage and high-grade tumors in the discovery stages (to focus on events linked to early ccRCC development) and access to data on smoking status (the other widely acceptable epidemiological risk factor for ccRCC).

In summary, we report the first evidence of a somatic alteration in ccRCC that appears to be specifically linked to obesity. Specifically, that the obese versus non-obese fold change for the ENRAGE gene is significantly different in ccRCC tumor tissues in comparison with the patient-matched normal kidney tissues. In support of ENRAGE expression as a somatic event that defines a unique subset of ccRCCs, we also demonstrated that ENRAGE expression is higher in ccRCC tumors that are wild-type for the VHL gene and those with poorer overall survival. If our preliminary results are further validated using a larger and more robust study design, they would have clear implications for advancing our understanding of ccRCC carcinogenesis as well as inform novel methods of prevention.

Supplementary material

Supplementary Tables 1–3 and Figures 1 and 2 can be found at http://carcin.oxfordjournals.org/

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References


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