MicroRNA target site polymorphisms in the VHL-HIF1α pathway predict renal cell carcinoma risk

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Abstract

Renal cell carcinoma (RCC) accounts for ~4% of all human malignancies and is the 9th leading cause of male cancer death in the United States. The purpose of this study was to determine the effect of variation within microRNA (miRNA) binding sites of genes in the VHL-HIF1α pathway on RCC risk. We identified 429 miRNA binding site single nucleotide polymorphisms (SNPs) in 102 pathway genes and assessed 53 tagging-SNPs for 31 of these genes for risk in a case-control study consisting of 894 RCC cases and 1,516 controls. Results showed that five SNPs were significantly associated with RCC risk. The most significant finding was rs743409 in *MAPK1*. Under the additive model, the variant was associated with a 10% risk reduction (OR: 0.90, 95% CI, 0.77-0.98). Other significant findings were for SNPs in *CDCP1*, *TFRC*, and *DEC1*. Cumulative effects analysis showed that subjects carrying four or five unfavorable genotypes had a 2.14-fold increase in risk (95% CI, 1.03-4.43, *P* = 0.04) than those with no unfavorable genotypes. Potential higher-order gene-gene interactions were identified and categorized subjects into different risk groups. The OR of the high-risk group defined by two SNPs: *CDCP1*:rs6773576 (GG) and *DEC1*:rs10982724 (GG) was 4.46-times higher than that of low-risk reference group (95% CI, 1.31-15.08). Overall, our study provides the first evidence supporting a connection between miRNA binding site SNPs within the VHL-HIF1α pathway and RCC risk. These novel genetic risk factors might help identify individuals at high risk to enable detection of tumors at an early, curable stage.

Keywords

VHL-HIF1α pathway; microRNA; renal cell carcinoma

Introduction

Renal cell carcinoma (RCC) accounts for ~4% of all human malignancies and is the 9th leading cause of male cancer death in the United States [1]. In 2012, it is estimated that approximately 64,770 cases of kidney and renal pelvis cancers will be diagnosed, resulting in 13,570 deaths in the United States [1]. The incidence of RCC has been increasing by an estimated 2-3% annually for the past two decades [2]. The 5-year cumulative survival rate of patients with RCC was 70% and has remained unchanged for 10 years [3]. For patients with tumors that have spread to regional lymph nodes, the 5-year cancer-specific survival rate is

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63.3%, and once RCC has metastasized, the 5-year survival rate is 11.1% [4]. Because RCC is a "quiet" disease and typically diagnosed at an advanced stage when systemic chemotherapy has limited or no effectiveness [5,6], it is vital to develop new approaches for risk prediction and early detection to improve overall survival in RCC patients.

The VHL-HIF1α pathway has been well established as playing a major role in the pathogenesis of RCC. Mutations in this pathway, particularly in the tumor suppressor Von Hippel-Lindau (VHL), are associated with familial and sporadic renal cancer with VHL mutations detected in 61% of sporadic clear cell RCCs [7]. VHL promoter hypermethylation was also detected in more than 20% of sporadic clear cell RCC cases [8]. The main function of VHL is its E3-ubiquitin ligase activity which results in degradation of target proteins. Hypoxia inducible factor (HIF) is a critical downstream target of VHL. HIF is a basic helix-loop-helix transcription factor that consists of an oxygen-sensitive α-subunit HIF-α, and a constitutively expressed beta-subunit, HIF-β (encoded for by *ARNT*). HIF is normally bound to VHL and then degraded by an oxygen dependent ubiquitin-proteasome-mediated pathway [9]. Under normal conditions, the HIF-α subunit is hydroxylated by specific prolyl-hydroxylases and targeted for rapid degradation by VHL [10,11]. In a hypoxic environment or in the absence of functional VHL, HIF-1α is not degraded and translocates to the nucleus, where it dimerizes with HIF-β to form active HIF [10]. Besides VHL, cytoplasm HIF-1α is also regulated by the p53-MDM2 complex, MAPKs, AKT, and HSP90 [12,13]. HIF is involved in the regulation of many biological processes that facilitate both oxygen delivery and oxygen deprivation adaptation, as well as cell proliferation and angiogensis through vascular endothelial growth factor (VEGF), platelet-derived growth factor β (PDGF-β) and transforming growth factor-α (TGF-α) and metastasis through the repression of E-cadherin [14-16]. Research has also demonstrated that HIF inhibition is sufficient to decrease RCC tumor formation [17]. Inhibition of VEGF, PDGF, and TGF-α by sorafenib, axitinib, sunitinib, and bevacizumab are the basis for current treatment of metastatic RCC [18,19]. In addition, inhibition of HIF by PI3K/Akt/mTOR pathway inhibitors is another strategy under development [18,20].

MicroRNAs (miRNA) are a class of small noncoding RNA molecules 19-22 nucleotides in length. MiRNAs regulate gene expression by binding to target gene 3′ untranslated regions (3′UTR). As of November 2011, 1,527 human miRNA genes had been identified and deposited in the miRNA database (http://www.mirbase.org). Each miRNA may regulate hundreds of different protein-coding genes and depending on context it can function as either an oncogene or tumor suppressor. Previous studies have demonstrated that mutations, deletions, amplifications, over expression, and epigenetic silencing of miRNAs are involved in cancer [21]. MiRNAs regulate gene expression through complementarity to targeted 3′ UTR [22]. Recently, several genes known to play a role in RCC pathogenesis (mTOR, VHL, HIF-1α, PDGFβ) were detected as potential targets of miRNAs that were dysregulated in RCC [23]. Recently, our group identified genetic variations within miRNAs and miRNA-processing genes that modify RCC risk, survival, and recurrence [24,25]. However, there has been no report regarding the effect of genetic variation within miRNA binding sites of important cellular pathways involved in RCC pathogenesis on RCC risk.

Polymorphisms in miRNA binding sites have attracted attention because they can create a new binding site, disrupt an existing site, or change miRNA binding affinity. These alterations can thus affect normal miRNA function, potentially leading to cancer development. The Polymorphism in miRNA Target Site (PolymiRTS) database scans for SNPs located in the 3′UTR of each gene and predicts the effect on putative miRNA target sites [26]. We utilized the wealth of information in this database to scan more than one hundred genes that function in the VHL-HIF1α pathway for variants that potentially alter miRNA binding sites. The tagging SNPs for these variants were then assessed for RCC risk

in a large case-control population. To our knowledge, these variants have not been previously assessed for association with cancer development.

Patients and Methods

Study Population and Epidemiologic Data

The proposed study was carried out within the framework of the ongoing case-control study of RCC which has been recruiting incident cases from The University of Texas MD Anderson Cancer Center in Houston, Texas since 2002. All patients were residents of Texas, have histologically confirmed RCC and were previously untreated by chemotherapy or radiotherapy. There are no age, sex, ethnicity, and cancer stage restrictions for participation in the study. Population-based controls are recruited using an established modified random digit dialing protocol. The control participants were frequency matched to the cases in terms of age, sex, ethnicity, and county of residence. We also included controls from an ongoing bladder cancer case-control study who were involved in a previously published GWAS of bladder cancer [27]. For the current analysis, we included 894 cases and 1,156 controls who had previously been included in our genome-wide scan analysis [28]. This population was restricted to non-Hispanic whites to limit the confounding effect from population stratification. The study has been approved by the Institutional Review Board of MD Anderson Cancer Center. Written informed consent has been obtained from all study participants.

Epidemiologic data were collected through in-person interview using a pre-tested risk factor questionnaire conducted by trained MD Anderson staff personnel. Following the interview, a 40-mL peripheral blood sample was obtained for DNA extraction and storage.

Selection of miRNA binding site tagging SNPs in VHL-HIFα pathway genes

We utilized Ingenuity systems pathway analysis (Ingenuity Systems, Inc. Redwood City, CA) and PubMed to identify an extensive list of VHL-HIFα pathway-related genes. A total of 102 candidate genes were selected for analysis with RCC risk. Next, we scanned for miRNA binding site SNPs within the 3′UTR of these genes using the PolymiRTS database (http://compbio.uthsc.edu/miRSNP/). We further selected SNPs based on a minor allele frequency (MAF) greater than 0.01 as reported in dbSNP (http://www.ncbi.nlm.nih.gov/projects/SNP/). The complete set of 429 SNPs were screened with software package SNAP (http://www.broadinstitute.org/mpg/snap/) to identify tagging SNPs with r^2 threshold larger than 0.8. In the case of multiple binding site SNPs within the same haplotype block (defined by the linkage disequilibrium coefficient $r^2 > 0.8$), only one tagging SNP was selected. After SNAP screening of this set of SNPs, 53 tagging SNPs in 31 genes were present on the Illumina 610 array (Illumina, San Diego, CA). We then extracted the genotyping information from our existing data [28] generated using this chip to perform statistical analysis.

Statistical analysis

Lab and epidemiological data was merged and cleaned using system files created with the SAS software package (Cary, NC). Most analyses were performed using STATA 10.0 (Stata Corporation, College Station, TX) and HelixTree (Golden Helix, Bozeman, MT). Distributions of characteristics between cases and controls were evaluated by the χ^2 test (for categorical variables) or student's t-test (for continuous variables with normal distribution). Multivariate unconditional logistic regression model was applied to calculate odds ratios (ORs) and 95% confidence intervals (95% CIs) for analyzing the main effects of selected SNPs on RCC risk under three different genetic models of inheritance: dominant, recessive, and additive. The best-fitting model was the one with the smallest P value. Bootstrap

resampling was performed 1000 times to internally validate the results from our analyses [29]. The combined effects of unfavorable genotypes analysis included those SNPs showing statistical significance in the main analysis ($P < 0.05$). Higher-order gene–gene interactions were evaluated using Classification and Regression Tree (CART) analysis implemented in the HelixTree software. We also performed 10,000 bootstrap runs to internally validate our CART analysis results. All statistical analyses were two-sided.

Results

Characteristics of the study population

This study included 894 cases and 1,516 controls, who were all non-Hispanic whites (Table 1). The mean age was 63.23 ± 10.91 years for controls and 59.69 ± 10.69 for the cases, which was a significantly different between the two groups ($P < 0.001$). A significant difference in sex was also observed ($P < 0.001$) with the controls having a higher number of male participants. There was no significant difference in terms of smoking status between these two groups ($P > 0.05$).

Main effects of miRNA binding site genetic variation in VHL-HIFα pathway genes

Fifty-three tagging SNPs for miRNA binding site variants were identified from 31 selected genes (Table 2). Among these tagging SNPs, five were significantly associated with RCC risk ($P < 0.05$, Table 3). The most significant associations were observed for two SNPs in *MAPK1* (mitogen-activated protein kinase 1). Rs743409 was associated with a 10% reduction in risk (HR, 0.90, 95% CI 0.77-0.98; $P = 0.02$) under the additive model. *MAPK1*:rs9607241 resulted in a 14% increase in risk (HR, 1.14, 95% CI, 1.02-1.29, $P = 0.03$) under the additive model. In addition, rs6773576 in *CDCP1* (CUB domain-containing protein 1) resulted in an 18% increase in risk (HR, 1.18, 95% CI, 1.01-1.37, $P = 0.03$) under the additive model, *TFRC* (transferrin receptor):rs406271 resulted in an 88% increase in risk (HR, 1.88, 95% CI, 0.77-1.00, $P = 0.04$) under the additive model, and rs10982724 in *DEC1* (deleted in esophageal cancer 1) resulted in an greater than 2-fold increase in risk (HR, 2.18, 95% CI, 1.01-4.74, $P = 0.05$) under the recessive model. Three of these five top SNPs (rs13943, rs1063311, and rs12947) had highly consistent results in bootstrap analysis for internal validation, with bootstrap P values < 0.05 for more than 800 of 1000 samplings (Table 3).

Cumulative effects of SNPs on RCC risk

To further assess the effects of miRNA binding site variants on RCC risk, we performed a cumulative analysis of these five SNPs identified as significant in the main effects analysis. The risk differed significantly among these five groups ($P_{\text{for trend}} < 0.001$; Table 4) with an increase in risk with an increase in the number of unfavorable genotypes. Compared with the reference group, subjects carrying four or five unfavorable genotypes had a 2.14-fold increase in risk (95% CI, 1.03-4.43, P = 0.04).

Higher-order gene-gene interactions

We next explored higher-order gene-gene interactions to determine whether or not complex interactions among these significant SNPs could further modulate RCC risk. The final tree structure identified several potential interactions among the top five SNPs (Figure 1). *CDCP1*:rs6773576, which tags rs12947 located in a binding site for hsa-miR-379, hsa-miR-380, hsa-miR-3924, hsa-miR-411, and hsa-miR-4495 was identified as the initial split, suggesting its potential predictive role for cancer risk. The final tree structure identified two terminal nodes with significantly different risk. Compared to patients with the low-risk genetic profile, those with the high-risk genetic profiles characterized by node 3 had a 1.39-

fold increased risk (95% CI, 1.02-1.90) and those in node 4 had a highly significant nearly 4.5-fold increase in risk (95% CI, 1.32-15.08) To provide internal validation of the tree structure generated by this analysis, we performed bootstrap analysis with 10,000 re-samplings. We found that these results were highly consistent (Figure 1 and Table 5).

Discussion

The incidence of RCC continues to rise over the past two decades and has been thought to be due to an increased incidental detection by the radiographic identification [30]. It has been well established that the VHL-HIF1α pathway plays an important role in renal tumorigenesis [10,11]. We identified 429 miRNA binding site SNPs in 102 VHL-HIF1α pathway genes, and evaluated 53 tagging SNPs for their associations with RCC risk. Our results identified five tagging SNPs significantly associated with RCC risk. CART analysis further revealed potential high-order gene-gene interactions and categorized subjects into different risk groups according to their specific polymorphic signatures. Our study provides the first molecular epidemiological evidence supporting a connection between miRNA binding site SNPs within the VHL-HIF1α pathway and RCC risk.

The top two significant SNPs tagged binding site SNPs in *MAPK1*, which highlights the important function of this gene in RCC risk (Table 3). MAPK1 (also known as ERK2) has been found to be significantly active in advanced RCC and activity levels can predict the onset of metastasis with localized disease [31].It has been shown that HIF-1α Ser-641/643 phosphorylation by p42 MAPK (ERK2) promotes HIF-1α nuclear accumulation and transcriptional activity [32]. MAPK1 has also been demonstrated to be a critical mediator of EPAS/HIF-2α activation [33]. In addition, MAPKK inhibitor PD98059 has been shown to block HIF-1α's transcriptional activity [34]. PolymiRTS database annotated the G allele of rs13943 as a potential binding site of hsa-miR-3145-5p, and the C allele as a potential binding site for hsa-miR-1288 and has-miR-3169. It follows that these binding sites and miRNAs changes are associated with MAPK1 activation changes which contribute to RCC risk. Alternatively, it is possible that these tagging SNPs may be linked to other causal variants in *MAPK1*.

In addition to *MAPK1* variants, tagging SNP: rs6773576 in *CDCP1* was also found significantly associated with RCC risk and with bootstrap *P* values < 0.05 for more than 800 of 1000 samplings (Table 3). It has been reported that induction of CDCP1 is regulated by HIF-1/2 and increases ccRCC migration by activation of PKCδ, and patient survival can be stratified by cell surface expression levels of CDCP1 [35]. Tagging SNP: rs6773576 tags miRNA binding site SNP: rs12947 in *CDCP1* and PolymiRTS database predicted that the A allele of rs12947 is a potential binding site for hsa-miR-379, hsa-miR-380, hsa-miR-3924, hsa-miR-411, and hsa-miR-4495. However, G allele of rs12947 destroys these binding sites without creating a new site. Same as those in *MAPK1*, it is probably that the binding site and miRNAs changes are associated with CDCP1 level change or linked to other causal variants in *CDCP1* that contribute to RCC risk.

We identified a significant gene-dosage effect for the five tagging SNPs that had significant main effects. Those with the highest number of risk genotypes had the highest risk of RCC, suggesting that additional variation within this key pathway involved in RCC development was detrimental and had a larger effect than any single variant. Furthermore, the magnitude of each individual SNP was modest, but cumulatively doubled the risk for individuals with four or five of these risk genotypes. This highlights the importance of assessing multiple SNPs within a shared pathway for risk assessment. Further studies will be needed to explore the biological basis for these findings and establish functional interactions that modulate RCC risk.

Again, within the framework of a pathway, we hypothesized that gene-gene interactions would further modulate risk of RCC. Indeed that is what we observed. Potential gene-gene interactions among three variants were observed with *CDCP1:* rs6773576 being the initial split in our CART analysis, suggesting that this variant was responsible for the most variation in risk. DEC1 is a hypoxia-regulated transcription factor that functions downstream of both CDCP1 and MAPK1. Individuals carrying the common genotype for *CDCP1*: rs6773576 and the variant genotype for *DEC1*: rs10982724 had significant almost 4.5-fold increase in risk, a much larger effect magnitude than that conferred by either variant individually. Further studies will be needed to explore the biological basis for these findings and establish functional interactions that modulate RCC risk.

In summary, we have performed analysis of miRNA binding site variants in VHL-HIF1α pathway genes and RCC risk. These data may offer some mechanistic assumptions, as well as providing genetic information for predicting those at risk and identify tumors in an early, curable stage. However, there are some limitations to this research. First, other SNPs in important regions (exons, introns, promoters, etc) were not included in this specific study. It is also possible that some miRNA binding site SNPs were not predicted by Targetscan and therefore were not included into our study. Although rare VHL mutations contribute to RCC risk, we did not have information on these mutations for our study participants. In addition, we limited our analysis in non-Hispanic Caucasians to control potential confounding by population stratification. Future replication studies in independent populations will be needed to verify some of our results. Finally, besides age, gender, and smoking status, other epidemiology risk factors for RCC have been identified or suspected as risk factors, such as overweight and obesity, kidney disease, hypertension, alcohol consumption, and dietary factors [36]. Due to lack of information regarding these factors in all of our study participants, we were unable to include them in our analysis. However, our previous study showed that such risk factors might not significantly affect the relationship between SNPs and RCC risk [37].

Nevertheless, the comprehensive query of the VHL-HIFα pathway polymorphisms and our large population (894 cases and 1516 controls) with detailed risk information provide substantial evidence for the involvement of these novel miRNA binding site SNPs as predictors or modulators of RCC risk. Together with other known epidemiology, clinical, and genetic factors, may contribute to a risk assessment model for identifying those at high risk.

Acknowledgments

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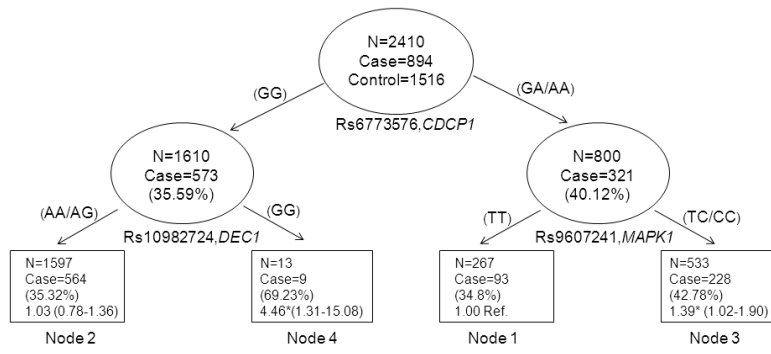
Figure 1. Higher-order gene-gene interactions and RCC risk

Values in each node denotes the case/control numbers with OR and 95% CI in parenthesis.
*Significant at $P < 0.05$

Table 1

Characteristics of study population (*N*= 2410, 894/1516)

Variables	Control (n %)	Case (n %)	*P* value
Age, mean(SD)	63.23 (10.91)	59.69 (10.69)	<0.001
Sex			
Male	1174 (77.44)	609 (68.12)	
Female	342 (22.56)	285 (31.88)	<0.001
Smoking status			
Ever	853 (56.27)	515 (57.61)	
Never	663 (43.73)	379 (42.39)	0.52

Table 2

Tagging SNPs of genes carrying miRNA binding site SNP (MAF > 0.01)

Gene name	Tagging SNP
APC	rs11950612
CASR	rs2134225
CASR	rs9740
CCDN1	rs649392
CCDN1	rs7178
CCND1	rs1789172
CCND1	rs9344
CDCP1	rs17077237
CDCP1	rs9863819
CITED2	rs1131431
CRKL	rs2266953
CRKL	rs2539918
CRKL	rs4822700
DEC1	rs10982724
EGLN3	rs1680699
EPAS1	rs10495933
ESR1	rs2459111
ESR1	rs3798577
ETS1	rs4937333
ETS1	rs6590330
ETS1	rs8705
HIF1A	rs12435848
HK2	rs10496197
HK2	rs943
HSP90AA1	rs7155973
HSPA4	rs4705990
IGF2	rs3802971
LDHA	rs10766473
MAPK1	rs2876980
MAPK1	rs743409
MAPK1	rs9607241
MMP2	rs1861320
NOCA1	rs9309308
NOTCH3	rs1044009
PGF	rs2268613
PI3K	rs7614305
POU5F1	rs6905862
RBX1	rs138354
TFRC	rs406271

Gene name	Tagging SNP
TFRC	rs570
TFRC	rs6773576
TGFA	rs10172814
TGFA	rs10496180
TGFA	rs11466297
TGFA	rs3771515
TGFA	rs488065
TGFA	rs549386
TSC1	rs10491534
TSC1	rs10491535
TSC1	rs2809244
VEGFA	rs3025033
VHL	rs1642742
IGF2	rs7115054

Table 3

List of significant genes carrying miRNA binding site polymorphisms

miRNA Binding Site SNP[*]	Tagging SNP	Gene	Genotype	Best Model[‡]	Case/Control			OR (95%CI)[†]	P value	No. Times in bootstrap sample P<0.05
					ww	wv	vv			
rs13943	rs743409	MAPK1	G/A	ADD	261/396	436/734	197/386	0.90 (0.77-0.98)	0.02	952
rs1063311	rs9607241	MAPK1	T/C	ADD	282/541	434/711	178/264	1.14 (1.02-1.29)	0.03	903
rs12947	rs6773576	CDCP1	G/A	ADD	573/1037	281/427	40/52	1.18 (1.01-1.37)	0.03	848
rs406271	rs406271	TFRC	A/G	ADD	425/665	389/675	80/176	0.88 (0.77-1.00)	0.04	662
rs3750505	rs10982724	DEC1	A/G	REC	706/1233	173/271	15/12	2.18 (1.01-4.74)	0.05	514

*
miRNA binding site SNPs and tagging SNPs are within the same haplotype block ($r^2 > 0.8$)

†
Adjusted by age, sex, smoking status.

‡
Best model: the model with smallest P value; ADD: additive model, REC: recessive model.

ww, homozygous wild-type genotype; wv, heterozygous variant genotype; vv, homozygous variant genotype

Table 4

Number of unfavorable genotypes and RCC risk

Number	Control (n %)	Case (n %)	OR (95% CI)[*]	P[*] value
0	29(1.91)	11(1.23)	1(reference)	
1	261(17.22)	128(14.32)	1.35(0.65-2.83)	0.42
2	275(18.14)	149(16.67)	1.52(0.73-3.17)	0.26
3	681(44.92)	396(44.07)	1.62(0.79-3.31)	0.19
4-5	270(17.81)	210(23.44)	2.14(1.03-4.43)	0.04
P for trend				<0.001

[*] Adjusted for age, sex, smoking status.

Table 5

Effect of terminal nodes derived from CART analysis on RCC risk

Node group	Control (n %)	Case (n %)	OR (95% CI)[*]	P^{*} value	Bootstrap (95% CI)
Low risk (node 1)	174 (65.17)	93 (34.83)	1(reference)		
Medium low risk (node 2)	1033 (64.68)	564 (35.32)	1.03(0.78-1.36)	0.84	0.78-1.37
Medium high risk (node 3)	305 (57.22)	228 (42.78)	1.39(1.02-1.90)	3.65×10^{-2}	1.02-1.89
High risk (node 4)	4 (30.77)	9 (69.23)	4.46 (1.32-15.08)	1.63×10^{-2}	1.47-18.87
P for trend				1.72×10^{-3}	

Note: Node groups are as shown in Figure 1.

[*] Adjusted for age, sex, smoking status.