Carcinogenesis vol.31 no.10 pp.1805–1812, 2010
doi:10.1093/carcin/bgq168
Advance Access publication August 23, 2010

Genetic variations in microRNA-related genes are associated with survival and recurrence in patients with renal cell carcinoma

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We took a polygenic approach to evaluate the effects of 41 potentially functional single-nucleotide polymorphisms (SNPs) in microRNAs (miRNAs)-related genes on survival and recurrence among renal cell carcinoma (RCC) patients. During a median follow-up of 21.8 months, among 316 RCC patients, 64 died and 56 developed recurrence. In single-SNP analysis, we identified seven SNPs significantly associated with RCC survival and five SNPs with recurrence. The most significant associations were SNPs in *GEMIN4* with the variant alleles of both rs7813 and rs910925 associated with 1.74-fold [95% confidence interval (CI) = 1.15–2.62] increased risk of death, whereas the variant allele of rs3744741 conferred a decreased risk of death [hazard ratio (HR) = 0.39; 95% CI = 0.19–0.77]. Several SNPs belonging to the pre-miRNA and were identified to be significantly associated with RCC recurrence. Haplotypes of *DICER* and *DROSHA* were also associated with altered patient survival and recurrence. More importantly, we observed cumulative effects of multiple SNPs on RCC survival. Compared with subjects carrying zero to two unfavorable genotypes, those carrying three to five and six and more unfavorable genotypes had an increased risk of death with a HR of 2.49 (95% CI = 1.24–5.00) and 6.66 (95% CI = 2.49–17.86), respectively, with significant dose–response trend (*P* for trend<0.001). As the first study of miRNA-related genetic polymorphisms on RCC clinical outcome, our results strongly suggested that miRNA-related SNPs may impact the recurrence and survival in RCC patients. Future investigation in larger populations and functional characterizations are necessary to validate these results.

Introduction

Renal cell carcinoma (RCC) represents the third leading cause of death among genitourinary malignancies. In 2009, ~57 760 new cases of kidney and renal pelvis cancers are expected and ~12 980 deaths will occur in the USA (1). RCC is the most lethal urological malignancy with >40% of patients eventually die of the disease (2,3). The 5 year cancer-specific survival rates for stages I to IV were 81, 74, 53 and 8%, respectively (4). The majority (80–90%) of RCC has the clear cell histology. Surgical resection remains the best curative therapy for RCC (5). However, after the curative nephrectomy, 20–40% patients will develop recurrence (6).

MicroRNAs (miRNA) are a group of endogenous, small non-coding RNA molecules of 22 nucleotides (7). To date, ~700 human miRNAs have been cataloged in the miRBase registry, with the total number predicted to be 1000 (8). It has been estimated that miRNAs regulate the expression of approximately one-third of human genes (9,10). MiRNAs are generated in a precisely coordinated two-step pathway. Most miRNAs reside in intergenic or intronic regions and are transcribed as a part of a long transcript through RNA polymerases II (11). These primary miRNA transcripts (pri-miRNA) are processed in the nucleus by the microprocessor machinery, which contains the Drosha RNase and the double-strand RNA-binding protein DGCR8 (12). A hairpin precursor miRNA molecule of 70–100 nucleotides (pre-miRNA) is then produced, which translocates to the cytoplasm through the assistance of RAN GTPase and Exportin 5 (XPO5), where it is further processed by a protein complex that includes DICER, TRBP, AGO1 and AGO2, leading to the production of mature miRNAs (13,14). The global or specific deregulation of key genes in the miRNA biogenesis pathway has been associated with malignant transformation (11,13). The mature miRNAs negatively affect the expression level of their target genes through binding to the 3′ untranslated region of the target genes at the posttranscriptional level.

With the increasing choices of treatment modalities that potentially improve survival, it is of great clinical benefit to predict clinical outcome of RCC patients. Large-scale profiling of miRNA expressions using microarray or real-time polymerase chain reaction techniques has revealed significant associations between miRNA expression signatures and the etiology and prognosis of various cancers (9,15–17). Moreover, identification of germ line genetic variants that predict RCC clinical outcome has become an increasingly promising approach that complements to somatic biomarkers. Although genetic polymorphisms have been widely implicated in cancer treatment response (18,19), such evidence is lacking for the miRNA-related genes. In this study, we tested the hypothesis that common sequence variants in genes of miRNA and of the miRNA biogenesis pathway affect RCC clinical outcomes. We evaluated the effects of 41 potentially functional miRNA-related single-nucleotide polymorphisms (SNPs) as well as took a polygenic approach to evaluate the cumulative effects of these SNPs on survival and recurrence among RCC patients.

Abbreviations: CI, confidence interval; HR, hazard ratio; miRNA, microRNA; RCC, renal cell carcinoma; SNP, single-nucleotide polymorphism.

Materials and methods

Study population

The study population and enrollment have been described previously (20,21). Briefly, incident RCC cases were recruited from The University of Texas M. D. Anderson Cancer Center in Houston, Texas. M. D. Anderson Cancer Center staff interviewers identified RCC cases through a daily review of computerized appointment schedules for the Departments of Urology and Genitourinary Medical Oncology. All cases were individuals with newly diagnosed histologically confirmed RCC. There was no age, gender, ethnicity or cancer stage restrictions on recruitment. Demographic and epidemiological information were collected by in-person interview. Data collected including age, gender, ethnicity, as well as occupation history, tobacco use history, medical history and family history of cancer. After the interview, a 40 ml blood sample was drawn into coded heparinized tubes and sent to the laboratory for molecular analyses. All study participants were followed on treatments, survival and tumor recurrence. Clinical and follow-up data were abstracted from medical records. The study end point was overall survival and recurrence. The study was approved by the Institutional Review Board of M. D. Anderson Cancer Center. All participants signed an informed consent prior to participation in the study. 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 >12 months before diagnosis was coded as a former-smoker. Current smokers were those who were currently smoking or had quit <12 months before diagnosis.

SNP selection and genotyping

The selection of genes and SNPs have been described previously in detail (22). We identified 41 potential functional polymorphisms: 24 SNPs in 11 genes in

the miRNA biogenesis pathway, 7 SNPs in 7 pre-miRNAs and 10 SNPs in 8 pri-miRNAs. All SNPs have a reported minor allele frequency of >0.01 in Caucasians and were located in exons, promoters (within 2 kb of the gene) or untranslated regions. When we selected SNPs in the miRNA biogenesis pathway, except for two *AGO1* SNPs (rs636832 and rs595961) located in introns, all other polymorphisms reside in functional regions, including exons, UTRs and promoters (within 2 kb of the genes). In the case of multiple potentially functional SNPs within the same haplotype block (defined by the linkage coefficient $r^2 > 0.8$), only one SNP was included. One exception is *GEMIN4* rs7813 because it is the only SNP in the miRNA biogenesis pathway that has been reported in previous studies to be significantly associated with cancer risk (21). For SNPs in pri-miRNAs but not in pre-miRNAs, because we identified >200 such SNPs with a minor allele frequency of >0.01 in Caucasians, we included 10 SNPs from 8 pri-miRNAs whose mature counterparts have been extensively implicated in cancer etiology or clinical outcome. The genotyping procedures were also described previously in detail (22). All polymorphisms were genotyped using the SNPlex assay according to the manufacturer's instructions (Applied Biosystems, Foster City, CA). Genotypes were called by GeneMapper software (Applied Biosystems) using a template file provided with each custom SNPlex assay. Internal quality controls and negative controls were used to ensure genotyping accuracy, and 5% of all samples were randomly selected and genotyped in duplicate with 100% concordance.

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 vital status as well as recurrence status. The Cox proportional hazard model was used to assess the effect of individual SNPs on overall survival, defined as the time from the date of surgical resection to the date of death or last follow-up. Hazard ratios (HRs) and 95% confidence intervals (CIs) were estimated by fitting the Cox model while adjusting for age, sex, smoking status, tumor stage, grade and treatments. Similarly, we used Cox proportional hazards to analyze recurrence-free survival time, defined as the time from surgery to recurrence or last follow-up. Kaplan–Meier curves and log-rank tests were used to assess the differences in survival and recurrence-free survival times by individual polymorphisms.

We performed a joint analysis by counting the number of putative unfavorable genotypes showing significant association with clinical outcomes ($P < 0.05$) in the Cox model. HRs and 95% CIs were calculated using multivariate Cox proportional hazard models after adjusting for appropriate variables. STATA software (version 10.0; STATA Corp., College Station, TX) was used for the above analyses. To take into account of the issue of multiple comparisons, we used the false discovery rate calculation based on the Benjamini–Hochberg method (23). We calculated the false discovery rate-adjusted *Q*-values at the 5% level to see whether the observed *P*-values remained statistically significant after adjusting for multiple comparisons.

Haplotype and diplotype frequencies were analyzed using the HelixTree Genetics Analysis Software (Golden Helix, Bozeman, MT). Haplotypes were inferred using the expectation–maximization algorithm implemented in the HelixTree software. The adjusted HRs and 95% CI for each haplotype and/or diplotype were calculated using multivariate Cox regression using the most abundant haplotype and/or diplotype as the reference group.

Survival tree analyses using recursive partitioning were performed to investigate higher order gene–gene interactions and to identify subgroups of individuals at higher risk of death or recurrence using a modified STREE program (http://peace.med.yale.edu/pub/stree/) (24). The tree starts with the root node that includes all the study participants and uses a log-rank statistic to select the optimal split that distinguishes patients into better and worse survival. The recursive procedure continues to produce subsequent nodes that are more homogeneous (with respect to survival) than the original node. The final model is a tree structure with many binary splits, and each terminal node represents a group of patients with different survival patterns based on distinct genotype combinations. HRs and 95% CIs were calculated for terminal nodes using multivariate Cox proportional hazard models after adjusting for appropriate variables.

Results

The study included 316 patients with RCC. During a median follow-up of 21.8 months, 64 patients died (Table I). There was no significant difference in the distribution of sex by survival status ($P = 0.94$). The mean ages of patients that survived and did not survive were 58.88 and 59.36 years, respectively ($P = 0.75$). There was no significant difference in the distribution of smoking status ($P = 0.41$). Among ever-smokers, pack-years of smoking did not differ significantly by survival status ($P = 0.64$). Among clinical variables, there were no significant differences in terms of histology ($P = 0.21$), radiotherapy ($P = 0.27$) or molecular targeted therapy ($P = 0.26$). However, there was a significant difference in survival by tumor grade ($P < 0.001$), stage ($P < 0.001$) and tumor size ($P < 0.001$). Furthermore, alive patients were less probably to have received cytokine treatment ($P < 0.001$) or chemotherapy ($P = 0.006$) than patients who had died.

During the same follow-up period, 56 patients developed recurrence, whereas 181 patients had no recurrence. Again, there was no significant difference in sex, age, ethnicity or smoking status by recurrence status. Significant differences were found between pack-years of smoking, tumor stage, grade, tumor size and whether patients ever received chemotherapy (Table I).

The associations between single SNPs and RCC survival were shown in Table II. Overall, there were six SNPs significantly associated with RCC survival and one SNP with borderline significance ($P = 0.055$). Dominant model was the best-fitting model for rs910924, rs3744741 and rs4968104 in *GEMIN4*; additive model was the best-fitting model for rs7813 and rs910925 in *GEMIN4*, as well as rs4919510 in *mir608*. One SNP, rs3742330 in *DICER*, was borderline associated with RCC survival. Note that the most significant associations were observed in SNPs in *GEMIN4*. In particular, the variant alleles of both rs7813 and rs910925 were each associated with 1.74-fold (95% CI = 1.15–2.62; $P = 0.009$) increased risk of death, whereas the variant allele of rs3744741 conferred a significantly decreased risk of death with a HR of 0.39 (95% CI = 0.19–0.77; $P = 0.007$). The associations remained significant after adjusting for multiple comparisons. Similar results were obtained when we restricted the analysis in clear cell histology (Table II).

The association between RCC recurrence and single SNPs was shown in Table III. A total of five SNPs showed significant association with RCC recurrence. Dominant model was the best-fitting model for rs2910164 in *mir146a*, rs11614913 in *mir196a-2* and rs5745925 in *mir631*. Additive model was the best-fitting model for rs4919510 in *mir608*; recessive model was the best-fitting model for rs6505162 in *mir423*. Similar results were obtained if the analysis was restricted in clear cell histology (Table III). One SNP, rs4919510 in *mir608*, exhibited significant association with both RCC recurrence and survival (Tables II and III) with HR of 1.88 (95% CI = 1.12–3.16; $P = 0.017$) and 1.61 (95% CI = 1.00–2.57; P = 0.048), respectively.

To further assess the cumulative effects of miRNA-related genetic variants on RCC survival, we did a joint analysis by including the seven SNPs showing a significant or borderline significant association in single-SNP analysis above. The unfavorable genotypes were defined as following: rs3742330 (WM and MM), rs910924 (WM and MM), rs7813 (WM and MM), rs910925 (WM and MM), rs3744741 (WW), rs4968104 (WM and MM), rs4919510 (WM and MM), where WW is the wild-type genotype, WM is the heterozygous genotype and MM is the homozygous variant genotype. In case of closely linked SNPs, only one SNP was used to be summed up with other variants. Compared with the reference group, subjects carrying zero to two unfavorable genotypes, those carrying three to five and six or more unfavorable genotypes had an HR of 2.49 (95% CI = 1.24–5.00; $P = 0.01$), 6.66 (95% CI = 2.49–17.86; $P < 0.001$), respectively (P for trend<0.001) (Table IV).

We performed similar joint analysis to assess the cumulative effects of SNPs on recurrence by including the five SNPs showing a significant association in single-SNP analysis: rs2910164 (WW), rs11614913 (WW), rs6505162 (WW + WM), rs4919510 (WM + MM) and rs5745925 (WM + MM). Again, only one SNP was used to be summed up in case of closely linked SNPs. We found that compared with the reference group, subjects carrying one or zero unfavorable genotype, subjects carrying two unfavorable genotypes were at 4.10-fold (95% CI = 1.29–13.06; $P = 0.017$) increased risk of recurrence and the risk further increased to 6.84-fold (95% CI = 2.08–22.57; $P = 0.002$) among subjects carrying three unfavorable genotypes. The risk was 25.06-fold (95% CI = 7.00–89.76; $P < 0.001$) among subjects carrying four or more unfavorable genotypes (P for trend<0.001) (Table IV).

Table I. Characteristics of study population[a]

	Dead ($N = 64$)	Alive ($N = 252$)	P-value	Recurrence ($N = 56$)	No recurrence ($N = 181$)	P-value
Sex, *N* (%)						
Male	42 (65.63)	164 (65.08)	0.94	40 (71.43)	115 (63.54)	0.28
Female	22 (34.38)	88 (34.92)		16 (28.57)	66 (36.46)	
Ethnicity, *N* (%)						
White	54 (84.38)	206 (81.75)	0.82	46 (82.14)	146 (80.66)	0.48
Hispanic	2 (3.13)	12 (4.76)		1 (1.79)	10 (5.52)	
Black	8 (12.50)	34 (13.49)		9 (16.07)	25 (13.81)	
Age, mean (SD)	59.36 (10.21)	58.88 (10.57)	0.75	59.36 (9.74)	59.37(10.83)	0.99
Smoking status, *N* (%)						
Never	28 (43.75)	133 (52.78)	0.41	31 (55.36)	93 (51.38)	0.63
Former	27 (42.19)	86 (34.13)		17 (30.36)	67 (37.02)	
Current	9 (14.06)	33 (13.10)		8 (14.29)	21 (11.60)	
Pack-years, mean (SD)	26.47 (31.39)	28.78 (23.82)	0.64	39.42 (33.20)	24.91 (21.32)	0.01
Stage, *N* (%)						
I	6 (9.38)	128 (53.33)	<0.001	4 (7.14)	111 (65.29)	<0.001
II	5 (7.81)	20 (8.33)		7 (12.50)	15 (8.82)	
III	19 (29.69)	60 (25.00)		36 (64.29)	40 (23.53)	
IV	34 (53.13)	32 (13.33)		9 (16.07)	4 (2.35)	
Grade, *N* (%)						
I	0 (0.00)	3 (1.19)	<0.001	0 (0.00)	2 (1.10)	<0.001
II	7 (10.94)	79 (31.35)		8 (14.29)	69 (38.12)	
III	22 (34.38)	126 (50.00)		23 (41.07)	90 (49.72)	
IV	35 (54.69)	38 (15.08)		25 (44.64)	14 (7.73)	
Unknown	0 (0.00)	6 (2.38)		0 (0.00)	6 (3.31)	
Tumor size, mean (SD)	9.30 (4.14)	6.03 (3.78)	<0.001	9.68 (3.58)	5.24 (3.39)	<0.001
Histology, *N* (%)						
Conventional	55 (85.44)	199 (78.97)	0.21	46 (82.14)	145 (80.11)	0.74
Other	9 (14.06)	53 (21.03)		10 (17.86)	36 (19.89)	
Cytokine treatment, *N* (%)						
Yes	15 (23.81)	6 (2.44)	<0.001	0	1 (0.56)	0.58
No	48 (76.19)	240 (97.56)		55 (100.00)	179 (99.44)	
Chemotherapy, *N* (%)						
Yes	11 (17.46)	16 (6.45)	0.006	6 (10.91)	5 (2.78)	0.012
No	52 (82.54)	232 (93.55)		49 (89.09)	175 (97.22)	
Molecular targeted therapy, *N* (%)						
Yes	7 (11.11)	17 (6.85)	0.26	1 (1.82)	3 (1.67)	0.94
No	56 (88.89)	231 (93.15)		54 (98.18)	177 (98.33)	
Radiotherapy, *N* (%)						
Yes	2 (3.17)	3 (1.22)	0.27	0 (0.00)	1 (0.56)	0.58
No	61 (96.83)	243 (98.78)		55 (100.00)	179 (99.44)	

[a]Numbers in some categories do not add up to total due to missing values

To further explore potential higher order interactions among SNPs, we performed a survival tree analysis. For overall survival, the tree structure resulted in three terminal nodes with a range of low to high-risk subgroups (Figure 1). The initial split was rs3742330 of *DICER*, followed by rs4919510 of *mir608*. We selected terminal node 1 (subjects carrying the wild genotype of both SNPs) as the reference group for our analyses. The HRs for terminal nodes 2 (subjects carrying wild-type genotype of rs3742330 and at least one variant allele of rs4919510) and 3 (subjects carrying at least one variant allele of rs3742330) were 2.45 (95% CI = 1.31–4.59; $P = 0.005$), 3.54 (95% CI = 1.47–8.53; $P = 0.005$), respectively (Figure 1). We also performed the survival tree analysis to assess gene–gene interactions that affect RCC recurrence (Figure 2). The analysis resulted in three terminal nodes as defined primarily by two SNPs, rs4919510 in *mir608* and rs11614913 in *mir196a-2*. When using terminal node 1 (subjects carrying wild-type genotype of rs4919510) as the reference group, we found that the HRs for terminal nodes 2 (subjects carrying at least one variant allele of the rs4919510 and subjects carrying the wild-type genotype of rs11614913) and 3 (subjects carrying at least one variant allele of the rs4919510 and subjects carrying at least one variant allele of rs11614913) were 5.74 (95% CI = 2.58–12.78; $P < 0.001$) and 1.05 (95% CI = 0.46–2.42; $P = 0.91$), respectively (Figure 2).

In haplotype analysis, only haplotypes of *DICER* showed significant association with RCC survival. Specifically, compared with the AT haplotype (in order of rs3742330 and rs13078), the haplotype AA had a HR of 1.51 (95% CI = 0.99–2.31) and the haplotype GA was associated with increased HR of 2.04 (95% CI = 1.00–4.15) (Table V). Similarly, in diplotype analysis, using the AT-AT as the reference group, the diplotype AT-GT was associated with a 2.86-fold increased risk (95% CI = 1.11–7.34; $P = 0.03$) and the diplotype AA-AA was at 3.48-fold increased risk (95% CI = 1.21–9.97; $P = 0.02$). No significant associations were observed in other diplotypes. Regarding the association between haplotype/diplotype and recurrence, only haplotypes/diplotypes of *DROSHA* showed significant associations. Compared with the CG haplotype (in order of rs10719 and rs6877842), a 57% reduction in recurrence risk was observed for the CC haplotype (HR = 0.43; 95% CI = 0.22–0.87; $P = 0.02$) and the HR was 0.50 (95% CI = 0.27–0.90; $P = 0.02$) for the TG haplotype. Compared with the CG-CG diplotype, the diplotype CG-CC had a HR of 0.34 (95% CI = 0.12–0.96; $P = 0.04$), the diplotype TG-TG had HR of 0.18 (95% CI = 0.04–0.94; $P = 0.04$) and the HR of the diplotype TG-CC was 0.075 (95% CI = 0.008–0.67; $P = 0.02$) (Table V).

Discussion

In this systematic evaluation of the influence of genetic variations in the miRNA biosynthesis and miRNA genes on the clinical outcome of RCC patients, we identified seven SNPs that were significantly

Table II. HRs of single SNPs and RCC survival

Gene	SNP	Alleles[a]	Minor allele frequency (dead/alive)	Best-fitting genetic model (All histology types)			Best-fitting genetic model (Clear cell carcinoma)		
				Model	HR (95% CI)[b]	*P*-value[c]	Model	HR (95% CI)[b]	*P*-value
Biogenesis pathway									
DROSHA	rs6877842	G/C	0.15/0.16	DOM	0.79 (0.40–1.55)	0.494	DOM	0.91 (0.43–1.92)	0.811
	rs10719	C/T	0.26/0.26	DOM	0.68 (0.36–1.30)	0.246	DOM	0.59 (0.29–1.21)	0.151
DGCR8	rs417309	G/A	0.05/0.07	DOM	1.04 (0.42–2.54)	0.935	DOM	1.20 (0.48–2.99)	0.694
	rs3757	G/A	0.20/0.24	REC	0.44 (0.10–1.91)	0.273	DOM	0.91 (0.46–1.77)	0.771
	rs1640299	G/T	0.50/0.48	DOM	1.55 (0.82–2.95)	0.179	ADD	1.21 (0.77–1.89)	0.406
XPO5	rs11077	A/C	0.52/0.42	ADD	1.37 (0.91–2.05)	0.130	DOM	1.85 (0.89–3.83)	0.099
RAN	rs14035	C/T	0.29/0.28	ADD	1.25 (0.80–1.94)	0.324	REC	1.72 (0.62–4.79)	0.302
DICER	rs3742330	A/G	0.08/0.07	DOM	2.17 (0.98–4.79)	0.055	DOM	1.85 (0.78–4.39)	0.164
	rs13078	T/A	0.25/0.17	ADD	1.47 (0.96–2.27)	0.080	ADD	1.63 (0.98–2.72)	0.061
TRBP	rs784567	C/T	0.48/0.39	DOM	1.54 (0.79–2.99)	0.204	ADD	1.19 (0.79–1.80)	0.415
AGO1	rs636832	G/A	0.13/0.15	DOM	1.16 (0.58–2.29)	0.678	DOM	0.96 (0.43–2.11)	0.912
	rs595961	A/G	0.18/0.22	REC	1.63 (0.40–6.65)	0.494	ADD	0.88 (0.47–1.66)	0.699
AGO2	rs4961280	C/A	0.20/0.19	DOM	1.15 (0.63–2.10)	0.644	ADD	1.14 (0.66–1.96)	0.637
GEMIN4	rs910924	C/T	0.22/0.23	DOM	**2.04 (1.11–3.76)**	**0.022**	DOM	**2.27 (1.13–4.57)**	**0.021**
	rs2740348	G/C	0.16/0.15	DOM	1.59 (0.87–2.90)	0.132	DOM	1.39 (0.72–2.70)	0.325
	rs7813	T/C	0.37/0.36	ADD	**1.74 (1.15–2.62)**	**0.009**	ADD	**1.71 (1.08–2.70)**	**0.021**
	rs910925	G/C	0.37/0.37	ADD	**1.74 (1.15–2.62)**	**0.009**	ADD	**1.71 (1.08–2.70)**	**0.021**
	rs3744741	C/T	0.16/0.18	DOM	**0.39 (0.19–0.77)**	**0.007**	DOM	**0.40 (0.18–0.85)**	**0.018**
	rs1062923	T/C	0.19/0.16	DOM	0.75 (0.40–1.42)	0.379	DOM	0.83 (0.41–1.68)	0.604
	rs4968104	T/A	0.21/0.21	DOM	**1.88 (1.03–3.42)**	**0.040**	DOM	**2.01 (1.02–3.98)**	**0.044**
GEMIN3	rs197414	C/A	0.12/0.14	DOM	0.59 (0.30–1.17)	0.134	DOM	0.62 (0.30–1.26)	0.184
	rs197388	T/A	0.21/0.21	ADD	0.88 (0.54–1.43)	0.611	REC	0.28 (0.03–2.59)	0.259
	rs197412	T/C	0.40/0.45	ADD	0.70 (0.47–1.05)	0.087	ADD	**0.62 (0.40–0.98)**	**0.041**
HIWI	rs1106042	G/A	0.02/0.06	DOM	0.49 (0.11–2.12)	0.341	DOM	0.48 (0.11–2.10)	0.333
Pre-miRNA									
mir146a	rs2910164	G/C	0.25/0.27	DOM	0.77 (0.43–1.40)	0.396	DOM	0.56 (0.29–1.08)	0.08
mir196a-2	rs11614913	C/T	0.34/0.37	REC	1.44 (0.66–3.16)	0.359	DOM	0.80 (0.43–1.50)	0.481
mir423	rs6505162	C/A	0.49/0.46	REC	0.95 (0.47–1.91)	0.886	REC	0.84 (0.39–1.81)	0.647
mir492	rs2289030	C/G	0.06/0.06	DOM	1.49 (0.58–3.82)	0.407	DOM	1.40 (0.51–3.87)	0.516
mir604	rs2368392	C/T	0.21/0.27	ADD	0.73 (0.43–1.24)	0.247	ADD	0.70 (0.40–1.23)	0.217
mir608	rs4919510	C/G	0.25/0.21	ADD	**1.61 (1.00–2.57)**	**0.048**	ADD	1.64 (0.96–2.79)	0.071
mir631	rs5745925	CT/—	0.10/0.09	DOM	0.77 (0.37–1.59)	0.481	DOM	0.65 (0.27–1.57)	0.335
Pri-miRNA									
let7f-2	rs17276588	G/A	0.04/0.03	DOM	0.33 (0.09–1.15)	0.081	DOM	0.41 (0.12–1.47)	0.172
mir26a-1	rs7372209	C/T	0.37/0.28	DOM	1.51 (0.86–2.66)	0.154	DOM	1.33 (0.70–2.50)	0.384
mir30a	rs1358379	A/G	0.06/0.03	DOM	1.34 (0.59–3.07)	0.482	DOM	1.19 (0.45–3.17)	0.721
mir30c-1	rs16827546	C/T	0.02/0.04	DOM	0.26 (0.03–2.11)	0.208	N/A	N/A	N/A
mir100	rs1834306	C/T	0.47/0.46	REC	0.78 (0.38–1.59)	0.491	REC	0.81 (0.37–1.77)	0.597
mir124-1	rs531564	C/G	0.16/0.12	DOM	1.57 (0.85–2.88)	0.150	DOM	1.42 (0.72–2.81)	0.307
mir219-1	rs107822	G/A	0.20/0.26	DOM	0.75 (0.40–1.42)	0.380	ADD	0.70 (0.36–1.37)	0.296
	rs213210	T/C	0.06/0.07	DOM	0.88 (0.34–2.28)	0.790	DOM	0.70 (0.24–2.03)	0.511
mir373	rs12983273	C/T	0.15/0.14	DOM	1.75 (0.96–3.18)	0.066	DOM	**2.04 (1.04–3.98)**	**0.038**
	rs10425222	C/A	0.08/0.05	DOM	2.04 (0.89–4.68)	0.09	DOM	1.94 (0.67–5.62)	0.220

[a]Major/minor alleles.
[b]Adjusted for age, sex, ethnicity, smoking status, stage, grade and treatments. *P*-value < 0.05 was in bold and used in the unfavorable genotype analysis.
[c]Underlined *P*-values remained significant after false discovery rate adjustment at 0.05 level.

(including one SNPs with borderline significance) associated with RCC survival and five SNPs with RCC recurrence. We have previously reported that potentially functional SNPs in miRNA-related genes were associated with the etiology of multiple malignancies including RCC (21). This is the first epidemiological study evaluating the effects of these SNPs on RCC clinical outcome.

For individual associations with RCC survival, the most significant SNPs were in *GEMIN4* (rs7813 and rs910925, rs3744741). The minor alleles of both rs7813 and rs910925 were associated with 1.74-fold increased death risk for RCC, whereas the minor allele of rs3744741 was associated with 61% reduction in risk. *GEMIN4* belong to a family of genes whose products are components of a motor neuron complex and are involved in pre-mRNA splicing and ribonucleoprotein assembly (25). Previously, we have reported that genetic variants of *GEMIN4* and *GEMIN3* genes were associated with renal, bladder and esophageal cancer risks (21,22,26). In particular, in our previous case–control study, we found that the SNP rs7813 was associated with decreased risk of developing RCC (21). However, in the current study, the same SNP conferred an increased death risk of RCC. In addition, in the current study, the SNP rs3744741 was associated with better RCC survival, but in our previous study (26), the same SNP conferred reduced risk of esophageal cancer. It is not unprecedented that the same genetic trait may have differential effect on cancer susceptibility and cancer prognosis/treatment response. For example, weaker DNA repair capacity was associated with increased cancer risk but was also linked to better cisplatin response due to less removal of DNA–drug adducts (27). Both rs7813 and rs910925 are non-synonymous SNPs in exon 1 of *GEMIN4*, whose minor alleles resulted in the amino acid substitution of cysteine to arginine and glycine to alanine respectively (dbSNP; http://www.ncbi.nlm.nih.gov/projects/SNP/index). Linkage analysis showed that the two SNPs were in linkage disequilibrium ($r^2 > 0.8$). Expression of these variant forms of GEMIN4 protein in hepatocellular cancer cells led to increased cellular proliferation and reduced apoptosis and DNA repair (28), suggesting a causal physiological role for these genetic variants. Besides *GEMIN* SNPs, borderline significant association was found in one SNP in *DICER*

Table III. HRs of single SNPs and RCC recurrence

Gene	SNP	Alleles[a]	Minor allelle frequency (recurrence/no recurrence)	Best-fitting genetic model (All histology types)			Best-fitting genetic model (Clear cell carcinoma)		
				Model	HR (95% CI)[b]	*P*-value[c]	Model	HR (95% CI)[b]	*P*-value
Biogenesis pathway									
DROSHA	rs6877842	G/C	0.13/0.14	DOM	0.52 (0.23–1.18)	0.118	DOM	**0.36 (0.13–0.98)**	**0.046**
	rs10719	C/T	0.21/0.26	ADD	0.61 (0.34–1.10)	0.102	ADD	0.63 (0.33–1.20)	0.161
DGCR8	rs417309	G/A	0.05/0.05	DOM	0.52 (0.17–1.65)	0.269	DOM	0.79 (0.24–2.55)	0.693
	rs3757	G/A	0.17/0.24	DOM	0.61 (0.30–1.22)	0.163	DOM	0.57 (0.25–1.30)	0.182
	rs1640299	G/T	0.45//0.51	DOM	0.95 (0.49–1.86)	0.889	DOM	0.86 (0.40–1.87)	0.708
XPO5	rs11077	A/C	0.45/0.44	DOM	0.57 (0.29–1.13)	0.109	DOM	**0.36 (0.16–0.85)**	**0.020**
RAN	rs14035	C/T	0.28/0.29	DOM	0.55 (0.27–1.11)	0.097	DOM	0.50 (0.23–1.09)	0.080
DICER	rs3742330	A/G	0.04/0.06	DOM	0.75 (0.23–2.45)	0.640	DOM	0.52 (0.13–2.04)	0.348
	rs13078	T/A	0.14/0.19	DOM	0.77 (0.39–1.55)	0.468	DOM	0.61 (0.26–1.41)	0.244
TRBP	rs784567	C/T	0.47/0.40	DOM	0.79 (0.37–1.68)	0.534	REC	1.31 (0.60–2.86)	0.493
AGO1	rs636832	G/A	0.10/0.15	DOM	0.82 (0.32–2.11)	0.680	DOM	0.69 (0.24–1.99)	0.497
	rs595961	A/G	0.16/0.22	REC	3.52 (0.88–14.02)	0.075	REC	3.65 (0.71–18.68)	0.120
AGO2	rs4961280	C/A	0.20/0.20	REC	2.74 (0.30–24.66)	0.369	DOM	0.62 (0.27–1.47)	0.280
GEMIN4	rs910924	C/T	0.26/0.23	DOM	1.53 (0.76–3.09)	0.234	DOM	1.27 (0.54–3.02)	0.585
	rs2740348	G/C	0.16/0.15	DOM	0.88 (0.42–1.84)	0.740	DOM	1.15 (0.50–2.64)	0.744
	rs7813	T/C	0.41/0.36	REC	1.69 (0.67–4.28)	0.268	ADD	1.38 (0.80–2.37)	0.246
	rs910925	G/C	0.41/0.36	REC	1.69 (0.67–4.28)	0.268	ADD	1.38 (0.80–2.37)	0.246
	rs3744741	C/T	0.16/0.17	DOM	0.62 (0.30–1.27)	0.190	DOM	0.67 (0.28–1.57)	0.354
	rs1062923	T/C	0.16/0.15	DOM	1.09 (0.50–2.38)	0.837	DOM	0.88 (0.33–2.35)	0.801
	rs4968104	T/A	0.23/0.21	DOM	1.63 (0.85–3.12)	0.144	DOM	1.30 (0.55–3.05)	0.546
GEMIN3	rs197414	C/A	0.12/0.13	DOM	0.72 (0.30–1.71)	0.46	REC	0.70 (0.28–1.74)	0.44
	rs197388	T/A	0.22/0.21	DOM	0.57 (0.28–1.15)	0.12	REC	0.43 (0.19–0.98)	0.05
	rs197412	T/C	0.42/0.44	REC	0.51 (0.21–1.24)	0.137	REC	0.39 (0.14–1.08)	0.069
HIWI	rs1106042	G/A	0.05/0.07	DOM	0.52 (0.15–1.74)	0.288	DOM	0.54 (0.16–1.84)	0.325
Pre-miRNA									
mir146a	rs2910164	G/C	0.23/0.27	DOM	**0.47 (0.24–0.92)**	**0.029**	DOM	**0.42 (0.19–0.90)**	**0.026**
mir196a-2	rs11614913	C/T	0.34/0.38	DOM	**0.37 (0.19–0.73)**	**0.004**	DOM	**0.29 (0.14–0.64)**	**0.002**
mir423	rs6505162	C/A	0.43/0.48	REC	**0.39 (0.17–0.89)**	**0.026**	REC	0.46 (0.19–1.13)	0.091
mir492	rs2289030	C/G	0.06/0.08	DOM	0.79 (0.25–2.48)	0.692	DOM	0.58 (0.13–2.62)	0.476
mir604	rs2368392	C/T	0.22/0.28	REC	0.27 (0.04–1.99)	0.197	REC	0.28 (0.04–2.16)	0.223
mir608	rs4919510	C/G	0.29/0.22	ADD	**1.88 (1.12–3.16)**	**0.017**	ADD	**2.21 (1.24–3.92)**	**0.007**
mir631	rs5745925	CT/—	0.11/0.08	DOM	**3.93 (1.69–9.17)**	**0.002**	DOM	**4.62 (1.72–12.40)**	**0.002**
Pri-miRNA									
let7f-2	rs17276588	G/A	0.05/0.03	DOM	1.05 (0.32–3.43)	0.929	DOM	1.43 (0.41–4.98)	0.573
mir26a-1	rs7372209	C/T	0.26/0.29	DOM	0.84 (0.43–1.65)	0.622	ADD	0.67 (0.33–1.37)	0.274
mir30a	rs1358379	A/G	0.06/0.03	DOM	1.36 (0.44–4.18)	0.595	DOM	1.41 (0.36–5.56)	0.620
mir30c-1	rs16827546	C/T	0.00/0.04	N/A	N/A	N/A	N/A	N/A	N/A
mir100	rs1834306	C/T	0.40/0.49	REC	0.51 (0.21–1.24)	0.139	REC	0.53 (0.20–1.42)	0.206
mir124-1	rs531564	C/G	0.13/0.11	DOM	1.83 (0.83–4.07)	0.136	DOM	1.95 (0.83–4.59)	0.127
mir219-1	rs107822	G/A	0.27/0.27	DOM	1.85 (0.89–3.83)	0.097	DOM	2.18 (0.94–5.09)	0.071
	rs213210	T/C	0.08/0.07	DOM	1.47 (0.60–3.59)	0.401	DOM	1.47 (0.56–3.85)	0.433
mir373	rs12983273	C/T	0.14/0.14	DOM	1.68 (0.80–3.53)	0.174	DOM	1.23 (0.52–2.87)	0.640
	rs10425222	C/A	0.06/0.05	DOM	1.84 (0.64–5.27)	0.258	DOM	2.39 (0.69–8.26)	0.168

[a]Major/minor alleles.
[b]Adjusted for age, sex, ethnicity, smoking status, stage, grade and treatments. *P*-value < 0.05 was in bold and used in the unfavorable genotype analysis.
[c]Underlined P-values remained significant after false discovery rate adjustment at 0.05 level.

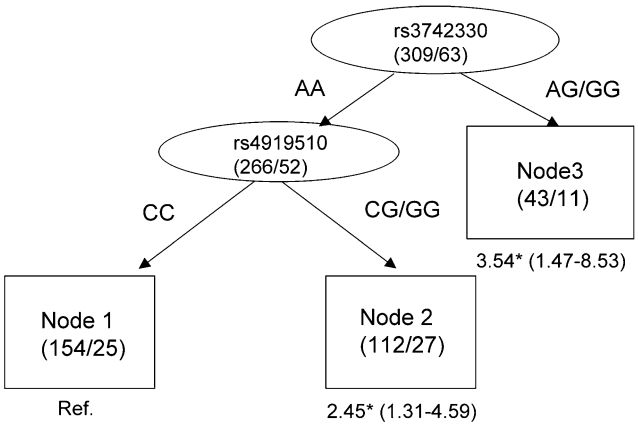
(rs3742330). Haplotype and diplotype analyses also showed that genetic variants of *DICER* were associated with increased risk of RCC mortality. Proteins encoded by *DICER* are members of the ribonuclease III family of double-stranded RNA endonucleases, which participate in RNA maturation and decay pathways (29). Lowered DICER1 protein expression was associated with incidence of ovarian cancer and advanced tumor stage (30). Using comparative genomic hybridization, Zhang *et al.* (31) found copy number changes in *DICER1*, Argonaute2 and other miRNA-related genes in breast and ovarian cancer as well as melanoma. There has been no report on DICER expression profiling in RCC. The correlation between DICER expression and DICER genotypes has yet to be elucidated. However, DICER expression has been examined in other cancers, such as ovarian (30), lung (32,33), esophageal (34) and prostate cancers (35). Decreased DICER expression was associated with advanced ovarian tumor stage and poor patient survival (30), whereas high DICER expression was a poor prognostic factor in patients with prostate and esophageal carcinoma (34,35). The differences in these reports have not been fully elucidated; however, these data suggest that the regulation and the effect of DICER expression may be tumor specific. Besides *GEMIN4* and *DICER*, one SNP in *mir608* (rs4919510) was significantly associated with both RCC survival and recurrence with the variant allele conferred significantly increased risk of survival and recurrence. The polymorphism of *mir608* (rs4919510) has been predicted by *in silico* algorithms to show differential binding to its target genes, which include *INSR* (insulin receptor) and *TP53* (36). It is possible that genetic variants of miRNA gene could alter its target gene specificities.

Individual SNPs associated with RCC recurrence were all pre-miRNA SNPs (Table 3), including *mir146a* (rs2910164), *mir196a-2* (rs11614913), *mir423* (rs6505162), *mir608* (rs4919510) and *mir631* (rs5745925). The *mir146a* gene has been implicated in the development of multiple cancers and the regulation of inflammation induced via the innate immune response (37). Decreased *mir146a* expression

Table IV. Number of unfavorable genotype and RCC clinical outcome

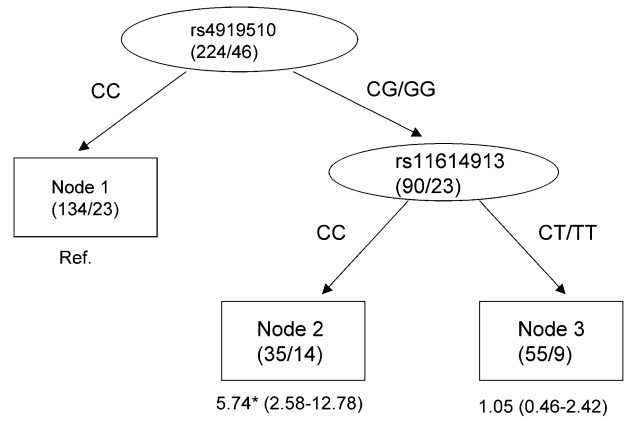
	Death	Alive	HR (95% CI)[a]	*P*-value
0–2	29 (50.00)	125 (52.52)	Ref.	
3–5	21 (36.21)	97 (40.76)	2.49 (1.24–5.00)	0.01
≥6	8 (13.79)	16 (6.72)	6.66 (2.49–17.86)	<0.001
P-trend				<0.001
	Recurrence	No recurrence		
0 or 1	4 (9.30)	43 (25.44)	Ref.	
2	16 (37.21)	56 (33.14)	4.10 (1.29–13.06)	0.017
3	13 (30.23)	57 (33.73)	6.84 (2.08–22.57)	0.002
≥4	10 (30.77)	13 (7.69)	25.06 (7.00–89.76)	<0.001
P-trend				<0.001

[a]Adjusted for age, sex, ethnicity, smoking status, stage, grade and treatments.



Note: The number under each node denotes HR with 95% confidence interval in parenthesis.
* means P<0.05

Fig. 1. Survival tree analysis of RCC survival.



Note: The number under each node denotes HR with 95% confidence interval in parenthesis.
* means significant at P<0.001

Fig. 2. Survival tree analysis of RCC recurrence.

has been associated with hormone refractory prostate cancer (38) and upregulation of this gene was found to suppress breast cancer metastasis (39). Variant allele of rs2910164 has been associated with increased risk for breast, ovarian and hepatocellular carcinomas (40,41), whereas in the current study, the variant allele was associated with decreased risk of RCC recurrence. More importantly, rs2910164 has been shown to be a functional polymorphism, which affects the production of the mature transcript as well as its binding to target mRNA, such as that of *BRCA1* (40,41).

Table V. *DICER* and *DROSHA* haplotype and diplotype and RCC outcome

	Death	Alive	HR (95% CI)[a]	*P*-value
DICER				
Haplotype				
A-T	87 (67.97)	385 (76.39)	Ref.	
A-A	31 (24.22)	85 (16.87)	1.51 (0.99–2.31)	0.06
G-A	10 (7.81)	34 (6.75)	**2.04 (1.00–4.15)**	**0.049**
Diplotype				
A_T-A_T	30 (46.88)	146 (57.94)	Ref.	
A_T-A_A	19 (29.69)	68 (26.98)	1.37 (0.72–2.61)	0.34
A_T-G_T	8 (12.50)	25 (9.92)	**2.86 (1.11–7.34)**	**0.03**
A_A-A_A	5 (7.81)	5 (1.98)	**3.48 (1.21–9.97)**	**0.02**
G_T-A_A	2 (3.13)	7 (2.78)	2.02 (0.40–10.14)	0.39
G_T-G_T	0 (0.00)	1 (0.40)	N/A	N/A
	Recurrence	No recurrence	HR (95% CI)[a]	*P*-value
DROSHA				
Haplotype				
C-G	77 (68.75)	223 (61.94)	Ref.	
C-C	15 (13.39)	50 (13.89)	**0.43 (0.22–0.87)**	**0.02**
T-G	19 (16.96)	85 (23.61)	**0.50 (0.27–0.90)**	**0.02**
T-C	1 (0.89)	2 (0.56)	0.87 (0.12–6.61)	0.9
Diplotype				
C_G-C_G	29 (51.79)	74 (41.11)	Ref.	
C_G-C_C	7 (12.50)	30 (16.67)	**0.34 (0.12–0.96)**	**0.04**
C_G-T_G	12 (21.43)	45 (25.00)	0.69 (0.28–1.67)	0.41
C_C-C_C	3 (5.36)	4 (2.22)	0.42 (0.07–2.40)	0.33
C_C-T_C	0 (0.00)	2 (1.11)	N/A	N/A
T_G-C_C	2 (3.57)	10 (5.56)	**0.075 (0.008–0.67)**	**0.02**
T_G-T_G	2 (3.57)	15 (8.33)	**0.18 (0.04–0.94)**	**0.04**
T_G-T_C	1 (1.79)	0 (0.00)	7.37 (0.64–84.14)	0.11

Bold numbers denote *P*-value < 0.05.
[a]Adjusted for age, sex, ethnicity, smoking status, stage, grade and treatments.

Our data showed that the variant T allele of the *mir196a-2* rs11614913 was associated with a decreased risk for RCC recurrence. High *mir196a* level has been shown to promote the oncogenic phenotype in colorectal cancer (42) and its expression is a potential biomarker of progression during transformation of Barrett's esophagus to adenocarcinoma (43). One of the known targets of *mir196a* is the annexin A1 gene (*ANXA1*) and suppression of *ANXA1* expression by *mir196a* led to increased cell proliferation and anchorage-independent growth and decreased apoptosis (44). Moreover, homozygous wild-type (CC) allele of rs11614913 in *mir196a* has been associated with increased expression of the mature transcript and decreased survival for non-small cell lung cancer in a Chinese population (45).

We have previously reported that rs6505162 of *mir423* was associated with decreased risk for esophageal cancer (26). In the current study, we found that variant A allele of rs6505162 was associated with 61% reduced risk for RCC recurrence. Increased expression of *mir423* has been associated with elevated risk for endometrial cancer (46). One of the putative target of *mir423* is a Kruppel-like factor 2 (*KLF2*) gene, which is involved in the regulation of endothelial proinflammatory function and angiogenesis by inhibiting hypoxia-inducible factor-1 alpha (*HIF-1α*) expression (46,47). Perhaps the variant allele of *mir423* has decreased capacity to target *KLF2* mRNA, which leads to increased inhibition of the inflammatory and angiogenic pathways and protects against risk of cancer recurrence. Further research is warranted to test the potential functional effects of these and other miRNA polymorphisms in RCC cells.

We also found that one SNP (rs5745925) in *mir631* was associated with increased risk of RCC recurrence. We previously reported that the same SNP was associated with increased risk of esophageal cancer (26). There has been no report on functional characterization of this SNP.

Several haplotypes and diplotypes of the *DROSHA* SNPs (rs6877842 and rs10719) showed significant association with RCC recurrence. As *DICER*, proteins encoded by *DROSHA* are members of the ribonuclease III family of double-stranded RNA endonucleases,

which participate in RNA maturation and decay pathways (29). It is interesting that the significant associations were not observed in single-SNP analysis but became evident in haplotype and diplotype analysis, suggesting that single SNPs in *DROSHA* may affect RCC recurrence jointly.

To explore the higher order interactions among the SNPs, we applied the survival tree analysis to further define high- versus low-risk subgroups. With this analytic approach, subgroups of individuals with different risk profile were identified based on SNP combinations. Because of the moderate sample size of this study, the number of subjects became small in terminal nodes. This analysis was therefore exploratory in nature and these results should be interpreted with caution.

Due to the relatively limited number of RCC cases, some of the SNPs we identified might be chance findings. However, the significant associations identified remained significant after adjusting for multiple comparisons. Furthermore, to increase detection power of the test, we took a pathway-based polygenic strategy to further elucidate the accumulative influences of multiple miRNA polymorphisms on RCC outcome. We identified an accumulative effect with an increasing number of unfavorable genotypes that occurred in a dose-dependent manner. Specifically, when multiple SNPs were analyzed together, a strong dose–response trend emerged, suggesting increased RCC death–recurrence risk with increasing number of adverse genotypes. Moreover, survival tree, haplotype and diplotype analyses indicated the joint effects of these miRNA genetic variants on RCC survival and recurrence, consistent with the polygenic nature of RCC clinical outcome and supporting the idea that polymorphisms of the miRNA-related genes may influence clinical outcome of renal cancer.

In conclusion, this is the first study that systematically evaluated the association between genetic variants in miRNA-related genes and RCC clinical outcome. Our results identified several putative variants that impact RCC survival and recurrence. The results from the cumulative analysis, haplotype analysis as well as higher order gene–gene interactions strongly suggested that these genetic variants may influence RCC clinical outcome jointly. However, the molecular mechanisms associated with these SNPs would depend on the functioanl impact of these SNPs on miRNAs and the interaction of specific miRNAs with target mRNAs in kidney tissues. Thus, future functional experiments are needed to elucidate the underlying molecular mechanisms associated with the SNPs. Nevertheless, validating current findings in independent patient populations is warranted.

Funding

National Cancer Institute (R01CA 98897, K07 CA134831).

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Received March 31, 2010; revised July 29, 2010; accepted July 30, 2010