Research Article

Cancer Prevention Research

Energy Balance, the PI3K-AKT-mTOR Pathway Genes, and the Risk of Bladder Cancer

Jie Lin[1], Jianming Wang[1], Anthony J. Greisinger[4], H. Barton Grossman[2], Michele R. Forman[1], Colin P. Dinney[2], Ernest T. Hawk[3], and Xifeng Wu[1]

Abstract

We evaluated the association between energy balance and risk of bladder cancer and assessed the joint effects of genetic variants in the mammalian target of rapamycin (mTOR) pathway genes with energy balance. The study included 803 Caucasian bladder cancer patients and 803 healthy Caucasian controls matched to cases by age (±5 years) and gender. High energy intake [odds ratio, 1.60; 95% confidence interval (95% CI), 1.23-2.09] and low physical activity (odds ratio, 2.82; 95% CI, 2.10-3.79) were each associated with significantly increased risk of bladder cancer with dose-response pattern ($P_{trend} < 0.001$). However, obesity (body mass index, ≥30) was not associated with the risk. Among 222 single nucleotide polymorphisms, 28 single nucleotide polymorphisms located in six genes of mTOR pathway were significantly associated with the risk. Further, the risk associated with high energy intake and low physical activity was only observed among subjects carrying a high number of unfavorable genotypes in the pathway. Moreover, when physical activity, energy intake, and genetic variants were analyzed jointly, the study population was clearly stratified into a range of low- to high-risk subgroups as defined energy balance status. Compared with subjects within the most favorable energy balance category (low energy intake, intensive physical activity, low number of unfavorable genotypes), subjects in the worst energy balance category (high energy intake, low physical activity, and carrying ≥7 unfavorable genotypes) had 21.93-fold increased risk (95% CI, 6.7-71.77). Our results provide the first strong evidence that physical activity, energy intake, and genetic variants in the mTOR pathway jointly influence bladder cancer susceptibility and that these results have implications for bladder cancer prevention. *Cancer Prev Res; 3(4); 505–17. ©2010 AACR.*

Introduction

Bladder cancer is the fourth most common cancer in men and the second most common urologic malignancy in the United States, with ~70,980 new cases and ~14,330 deaths in 2009 (1). Major risk factors for bladder cancer include male gender, older age, tobacco smoking, and occupational exposure to aromatic amines (e.g., in the dye and rubber industries; ref. 2). Energy balance, which is the ability to maintain body weight by balancing energy intake with energy expenditure, has been gaining increasing attention as an etiologic factor of cancers (3). A positive energy balance, in which energy intake exceeds energy expenditure over a prolonged time, leads to the development of overweight or obesity (4). The prevalence of obesity has reached epidemic levels in many parts of the world. In the United States, nearly two thirds of adults are overweight or obese (5). Epidemiologic studies have provided sufficient evidence to support obesity as a risk factor for cancers of the esophagus (6), colon and rectum (7), endometrium (8), and kidney (6, 9). Modulation of energy balance, through increased physical activity, has been shown in epidemiologic studies to reduce the risk of many cancers, including cancers of colon (10), endometrium (11), and breast (12). However, the association between physical activity and bladder cancer risk has been inconsistent in the literature (13–20) and previous studies have also generated conflicting results about the association between obesity and bladder cancer (14, 15, 20–34).

It is increasingly recognized that genetic susceptibility contributes to bladder carcinogenesis (35). Interindividual differences in genes that control the energy balance pathways may cause defects in energy metabolism and consequently contribute to increased cancer risk (36). Several hormones and growth factors serving as intermediate and long-term communicators of nutritional state have been implicated in both energy balance and carcinogenesis (37). Evidence suggests that many of these cellular

Authors' Affiliations: Departments of [1]Epidemiology and [2]Urology, [3]Division of Cancer Prevention and Population Sciences, The University of Texas M.D. Anderson Cancer Center; and [4]Kelsey Research Foundation, Houston, Texas

Corresponding Author: Xifeng Wu, Department of Epidemiology, Unit 1340, The University of Texas M.D. Anderson Cancer Center, 1155 Hermann Pressler Boulevard, Houston, TX 77030. Phone: 713-745-2485; Fax: 713-792-4657; E-mail: xwu@mdanderson.org.

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growth and metabolism genes involve signaling through the phosphatidylinositol 3-kinase/Akt/mammalian target of rapamycin pathway (PI3K-AKT-mTOR pathway or mTOR pathway; ref. 38). The mTOR pathway was first described ~15 years ago in studies investigating the mechanism of action of rapamycin (inhibitor of mTOR; ref. 39). mTOR is a member of the phosphoinositide-3-kinase–related kinase family, which is centrally involved in cell growth regulation, proliferation control, and cancer cell metabolism (40). Engagement of mTOR pathway allows both intracellular and environmental factors, such as energy supply and growth factors, to affect cell growth, proliferation, survival, and metabolism (37). Evidence suggests that a balanced energy from diet could modulate cellular signaling through cell surface receptors, affecting the activation or deactivation of multiple downstream genes within the mTOR pathway (38). Thus, genetic variations of the mTOR pathway–related genes and its interaction with energy balance may lead to the dysregulation of proliferation or decreased cell death, and subsequently increased cancer risk.

In this large case-control study, we evaluated whether there is an association between energy intake, physical activity, obesity, and bladder cancer risk. We also assessed whether genetic polymorphisms in the mTOR pathway–related genes interact with energy balance to modify bladder cancer risk.

Materials and Methods

Study population

This case-control study started patient recruitment in 1999 and is currently ongoing. Bladder cancer patients were recruited from the University of Texas M.D. Anderson Cancer Center and Baylor College of Medicine. The procedures for case recruitment and eligibility criteria were previously described (41). Briefly, all patients had

Table 1. Basic characteristics of cases and controls

Characteristics	Case n = 803, n (%)	Control n = 803, n (%)	P
Sex			
Men	640 (79.70)	639 (79.58)	0.951*
Women	163 (20.30)	164 (20.42)	
Age (y)			
Mean (SD)	64.73 (11.13)	63.82 (10.88)	0.098†
Smoking			
Never	212 (26.40)	355 (44.21)	<0.001*
Former	404 (50.31)	381 (47.45)	
Current	187 (23.29)	67 (8.34)	
Smoking pack-year			
Median (range)	38 (0.05-176)	22.5 (0.05-165)	<0.001‡
Energy intake (Kcal/d)			
Median (range)	2,218.95 (500.81-17,207.67)	1,991.39 (350.13-7,354.53)	<0.001‡
Energy intake category			
Low (<1,738 Kcal/d)	219 (28.82)	258 (33.25)	<0.001*
Medium (1,738-2,312 Kcal/d)	195 (25.66)	259 (33.38)	
High (≥2,313 Kcal/d)	346 (45.53)	259 (33.38)	
Physical activity			
Intensive (≥25)	129 (16.56)	265 (33.33)	<0.001*
Medium (9-24)	325 (41.72)	329 (41.38)	
Low (<9)	325 (41.72)	201 (25.28)	
BMI (kg/m^2)			
Mean (SD)	27.31 (4.59)	27.51 (4.65)	0.385†
BMI category			
Normal (<25 kg/m^2)	236 (30.26)	233 (29.23)	0.700*
Overweight (25-29 kg/m^2)	367 (47.05)	369 (46.30)	
Obese (≥30 kg/m^2)	177 (22.69)	195 (24.47)	

*χ^2 test.
†Student's t test.
‡Kruskal-Wallis test.

Table 2. Association between mTOR pathway genetic polymorphisms and bladder cancer

Gene	SNP*	Alleles (major/minor)	MAF		Best fitting model			$P_{H\text{-}W}$†
			Case	Control	Model	OR (95% CI)‡	*P*	
AKT3	rs2994329	G/A	0.20	0.20	Recessive	2.11 (1.21-3.67)	0.008	0.262
RHEB	rs717775	A/C	0.30	0.28	Recessive	1.60 (1.09-2.36)	0.016	0.361
RPS6KA5	rs7155799	G/A	0.22	0.24	Recessive	0.57 (0.35-0.94)	0.029	0.612
IRS2	rs9515120	G/A	0.07	0.05	Dominant	1.57 (1.12-2.19)	0.009	0.889
TSC2	rs2073636	G/A	0.41	0.37	Additive	1.18 (1.01-1.38)	0.036	0.721
RAPTOR	rs11653499	G/A	0.31	0.26	Additive	1.22 (1.03-1.44)	0.019	0.662
RAPTOR	rs7212142	G/A	0.43	0.38	Recessive	1.36 (1.02-1.82)	0.037	0.463
RAPTOR	rs7211818	A/G	0.24	0.21	Recessive	2.16 (1.35-3.47)	0.001§	0.637
RAPTOR	rs7208536	G/A	0.28	0.24	Recessive	1.86 (1.20-2.89)	0.005	0.274
RAPTOR	rs4969444	G/A	0.20	0.17	Recessive	2.16 (1.22-3.82)	0.009	0.744
RAPTOR	rs2048753	G/A	0.27	0.24	Additive	1.22 (1.02-1.45)	0.030	0.513
RAPTOR	rs2672890	G/A	0.18	0.20	Dominant	0.79 (0.63-0.99)	0.044	0.542
RAPTOR	rs9897968	G/A	0.21	0.23	Dominant	0.79 (0.63-0.98)	0.036	0.823
RAPTOR	rs1877926	G/A	0.35	0.33	Recessive	1.43 (1.02-1.99)	0.036	0.886
RAPTOR	rs2271612	A/G	0.49	0.47	Recessive	1.39 (1.07-1.81)	0.013	0.033
RAPTOR	rs6420481	A/G	0.43	0.42	Recessive	1.36 (1.02-1.80)	0.034	0.135
RAPTOR	rs1062935	A/G	0.50	0.48	Recessive	1.29 (1.00-1.66)	0.047	0.219

Abbreviation: MAF, minor allele frequency.

*In *raptor* gene, we only listed one significant SNP per haplotype block with the smallest *P* value.

†HWE test among controls.

‡Adjusting for age, sex, tobacco smoking, BMI, energy intake, and physical activity.

§q-value, <0.1.

histopathologically confirmed bladder cancer and had received no prior chemotherapy or radiotherapy upon recruitment. There are no restrictions on recruitment by age, sex, race, or cancer stage. The control subjects were healthy individuals without prior history of cancer (except nonmelanoma skin cancer). They were recruited from the Kelsey-Seybold Clinic, the largest private multispecialty group practice in the Houston metropolitan area, with 18 clinics and >325 physicians. The majority of control participants were healthy individuals seen at the clinic for annual physical exams. Controls were frequency matched to the patients on age (±5 y), sex, and ethnicity. The Kelsey-Seybold Clinic staff distributed a brief questionnaire about the study to potential control subjects when they arrived for clinic appointments. The questionnaire was used to elicit the patients' willingness to be contacted by staff at M.D. Anderson and to collect preliminary demographic data for matching. The potential control subjects were contacted by telephone at a later date to confirm their willingness to participate in the study and to schedule an in-person interview at a Kelsey-Seybold Clinic convenient to the participant. The response rates for cases and controls were 92% and 76.7%, respectively (41). The study is approved by the Institutional Review Board of M.D. Anderson, Kelsey-Seybold Clinic, and Baylor College of Medicine. Due to the small number of minority respondents in our sample, we restricted the analysis to Caucasians (non-Hispanic whites) in this study.

Data collection

After obtaining written informed consent from all participants, trained staff interviewers at M.D. Anderson administered a risk factor questionnaire to all participants. Data collected included demographic characteristics (age, gender, ethnicity), occupational history, tobacco use history, medical history, and family history of cancer. In addition to the risk factor questionnaire, a 45-min food-frequency questionnaire was administered to assess dietary intake during the year before diagnosis for cases and the year before the interview for control subjects. The food frequency questionnaire was a modified version of the Health Habits and History Questionnaire developed by the National Cancer Institute. The questionnaire includes a semiquantitative food frequency list of food and beverage items, ethnic foods commonly consumed in the Houston area, an open-ended section, and dietary behaviors such as dining at restaurant and food preparation methods. The validity and reliability of the questionnaire has been documented (42). Daily energy intake was estimated by the type and portion size of the food and expressed as an average calorie intake per day.

During the interview, participants were asked their current weight and height, and their weight 1 and 5 y before the date of interview (or diagnosis date for cases). These variables were used to derive body mass index (BMI), calculated as weight in kilograms divided by height in meters squared. In the current study, we used the weight 5 y ago

Table 3. The association between energy balance and bladder cancer stratified by smoking status

Variables	Overall risk			Never smoker			Former smoker			current smoker			$P_{interaction}$
	Case/ control	OR (95% CI)*	P	Case/ control	OR (95% CI)*	P	Case/ control	OR (95% CI)*	P	Case/ Control	OR (95% CI)*	P	
Energy intake													0.033
Low	219/258	Reference		73/119	Reference		99/121	Reference		47/18	Reference		
Medium	195/259	0.95 (0.72-1.25)	0.733	49/120	0.67 (0.42-1.06)	0.086	113/114	1.35 (0.92-1.98)	0.128	33/25	0.62 (0.27-1.46)	0.275	
High	346/259	1.60 (1.23-2.09)	0.001	77/103	1.50 (0.96-2.36)	0.076	165/133	1.60 (1.11-2.32)	0.012	104/23	2.31 (1.02-5.22)	0.044	
	P_{trend}		<0.001			0.1			0.013			0.011	
Physical activity													0.189
Intensive	129/265	Reference		45/123	Reference		68/122	Reference		16/20	Reference		
Medium	325/329	1.87 (1.42-2.47)	<0.001	91/135	1.97 (1.24-3.14)	0.004	173/169	1.69 (1.16-2.47)	0.006	61/25	3.15 (1.32-7.49)	0.009	
Low	325/201	2.82 (2.10-3.79)	<0.001	71/92	2.17 (1.32-3.57)	0.002	148/87	2.87 (1.89-4.35)	<0.001	106/22	6.33 (2.68-14.97)	<0.001	
	P_{trend}		<0.001			0.003			<0.001			<0.001	
BMI category													0.687
Normal	236/233	Reference		59/100	Reference		110/113	Reference		67/20	Reference		
Overweight	367/369	0.95 (0.73-1.23)	0.674	98/163	0.85 (0.54-1.33)	0.477	195/175	1.11 (0.77-1.58)	0.577	74/31	0.64 (0.30-1.38)	0.257	
Obese	177/195	0.79 (0.59-1.07)	0.131	48/87	0.76 (0.45-1.27)	0.297	87/92	0.84 (0.55-1.28)	0.409	42/16	0.79 (0.33-1.86)	0.584	
	P_{trend}		0.136			0.296			0.443			0.565	

*Adjusting for age, sex, tobacco smoking, energy intake, BMI, and physical activity where appropriate.

Table 4. The association between energy balance and bladder cancer stratified by the number of unfavorable genotypes

Variables	0-4 unfavorable genotypes			5-6 unfavorable genotypes			≥7 unfavorable genotypes		
	Case/control	OR (95% CI)*	*P*	Case/control	OR (95% CI)*	*P*	Case/control	OR (95% CI)*	*P*
Energy intake									
Low	82/107	Reference		72/85	Reference		60/50	Reference	
Medium	63/124	0.75 (0.48-1.19)	0.223	68/80	1.07 (0.66-1.73)	0.790	61/48	1.19 (0.67-2.12)	0.548
High	116/126	1.31 (0.86-2.00)	0.212	135/92	1.66 (1.05-2.62)	0.031	87/38	2.11 (1.16-3.87)	0.015
P_{trend}			0.155			0.026			0.014
Physical activity									
Intensive	49/123	Reference		41/85	Reference		34/50	Reference	
Medium	109/146	1.82 (1.17-2.84)	0.008	116/113	2.00 (1.22-3.28)	0.006	95/59	2.01 (1.12-3.63)	0.020
Low	108/95	2.39 (1.49-3.81)	<0.001	128/68	3.72 (2.22-6.23)	<0.001	82/30	3.45 (1.76-6.76)	<0.001
P_{trend}			<0.001			<0.001			<0.001
BMI category									
Normal	82/111	Reference		70/70	Reference		75/43	Reference	
Overweight	125/173	1.02 (0.67-1.55)	0.933	143/121	1.22 (0.77-1.92)	0.399	95/62	0.72 (0.41-1.25)	0.241
Obese	59/81	1.00 (0.61-1.64)	0.992	71/75	0.83 (0.50-1.39)	0.482	43/34	0.54 (0.28-1.04)	0.067
P_{trend}			0.987			0.450			0.065

*Adjusting for age, sex, tobacco smoking, energy intake, BMI, and physical activity where appropriate.

to calculate BMI. Participants also reported the average amount of time they spent on five broad groups of activities during the previous year and during their adult life. Individual activities included active sports such as tennis or racquetball; physical exercises such as aerobics, weight training, jogging or running, swimming, walking (including walking for golf), cycling, gardening or yard work, hunting, and housework; and other strenuous exercises. A metabolic equivalent task (MET) value was assigned based on the energy cost of each group of activity (43, 44). MET was defined as the ratio of working metabolic rate to a standard metabolic rate, in which 1 MET corresponds to resting metabolic rate obtained during quiet sitting. The energy expenditure from physical activity was calculated as the MET value of each activity multiplied by the frequency of each activity and then summed across all activities. In this study, we estimated the frequency of each activity based on the reported activities in the year before the interview. Immediately after the interview, each participant donated a blood sample for molecular analysis.

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 mo before diagnosis (for cases) or before the interview (for controls) was classified as a former smoker. Current smokers were those who were currently smoking or quit <12 mo before diagnosis (for cases) or before the interview (for controls).

Single nucleotide polymorphism selection and genotyping

Single nucleotide polymorphism (SNP) selection procedures were previously described (45). Briefly, we compiled our gene list using the SNPs3D bioinformatic tools, which is a Web-based literature mining approach to select genes according to a set of user-defined query terms of human diseases or biological processes. Then, we performed a literature review to refine the gene list in the PI3K-AKT-mTOR pathway. We identified tagging SNPs from the HapMap database (http://www.hapmap.org). All selected SNPs met the following criteria: $r^2 \geq 0.8$, minor allele frequency of ≥0.05 in Caucasians, and within 10 kb upstream of the 5′ untranslated region and 10 kb downstream of the 3′ untranslated region of the gene. In addition, we included potentially functional SNPs (e.g., coding SNPs and SNPs in the untranslated region, promoter, and splicing site). We also supplemented SNPs in this pathway previously genotyped as part of our genome-wide association study (46). For genes with less importance, only potentially functional SNPs with minor allele frequency of >0.01 (e.g., coding SNPs and SNPs in the untranslated region, promoter, and splicing site) were selected.

Genotyping of the SNPs was done using the Illumina Infinium II Assay as previously described (45). Briefly, genomic DNA was isolated from peripheral blood using the QIAamp DNA Blood Maxi kit (QIAGEN) according to the manufacturer's protocol. The genotyping was done using Illumina's iSelect custom SNP array platform according to the manufacturer's Infinium II assay protocol (Illumina) with 750 ng of input DNA for each sample. All genotyping data were analyzed and exported using the BeadStudio software (Illumina). The average call rate for the SNP array was 99.7% (45).

Statistical analyses

Statistical analyses were done by using STATA 10.0 (College station). Distributions in categorical variables

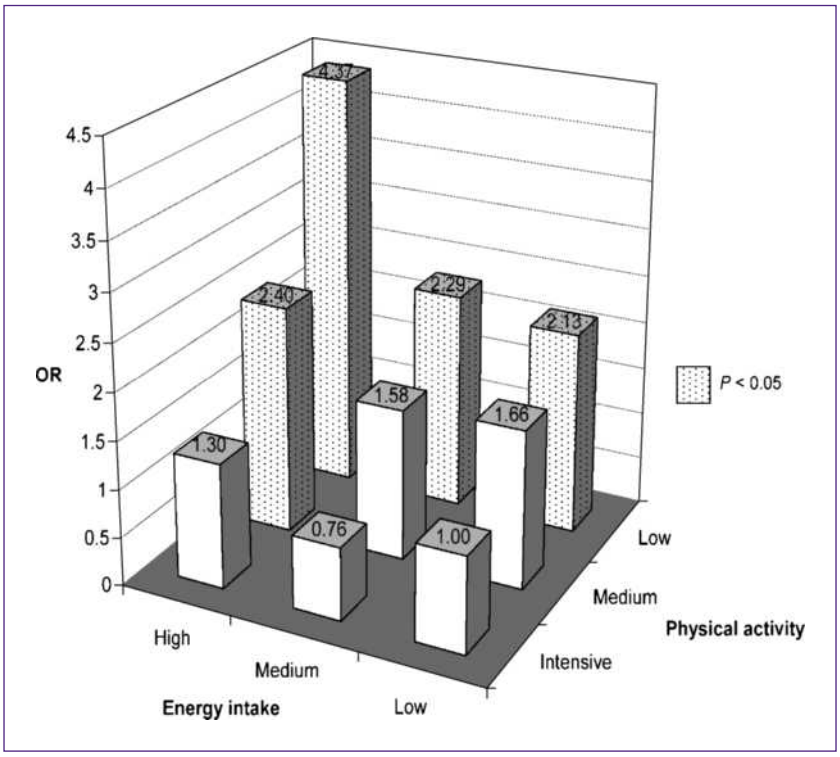

Fig. 1. Joint effects of energy intake and physical activity in the risk of bladder cancer. Number on top of each column is OR. Dotted columns, ORs with $P < 0.05$; blank columns, ORs that are not significant. The reference group is set as low physical activity and high energy intake.

between cases and controls were evaluated by the χ^2 test. Differences between cases and controls in continuous variables were tested using the Student's *t* test or the Kruskal-Wallis test (for continuous variables not normally distributed). The Hardy-Weinberg equilibrium was tested for each SNP using the goodness-of-fit χ^2 test to compare the observed with the expected frequency of genotypes in controls. BMI was divided into three groups: normal (BMI, <25 kg/m^2), overweight (BMI, ≥25 kg/m^2 but <30 kg/m^2), and obese (BMI ≥30 kg/m^2). For physical activity, the cutoff points were based on the tertile distribution of the MET scores in controls: low (<9), medium (≥9 but <25), and intensive (≥25). Total energy intake was also categorized into tertiles based on the cutoff points in controls: low (<1,738 Kcal/d), medium (≥1,738 Kcal/d but <2,313 Kcal/d), and high (≥2,313 Kcal/d). Multivariate logistic regression model was applied to calculate the odds ratios (OR) and their 95% confidence intervals (95% CI) associated with physical activity, obesity, and energy intake as well as the main effects of SNPs.

For SNP analysis, we tested three different genetic models, including the dominant, recessive, and additive model. The best fitting model was the one with the smallest *P* value among the three models. If the genotype counts for the homozygous variant genotype were less than five in cases and controls combined, we only considered the dominant model that had the highest statistical power. We calculated the q-value to account for multiple comparisons (47, 48). The q-value measured the proportion of false positive incurred (false discovery rate) when a particular test of SNP was called significant. To evaluate the modification effects from the genetic variants in the pathway, we summed up adverse genotypes (genotypes associated with significantly increased risk in the main effect analysis after adjustment for multiple comparisons) for each subject. In the case when multiple SNPs within a haplotype block were found to have significant main effect, only one most significant SNP with the smallest *P* value will be selected to be summed up with other variants in the pathway.

Interactions between variables were included in the multivariate model as cross-product terms and the significance was assessed using the likelihood ratio test. To explore higher order gene-gene and gene-environment interactions, we applied a recursive partitioning technique. The recursive partitioning was derived from the methodols of the Classification and Regression Tree analysis (CART). A tree-based model was created, which allowed identifying effect modifications between variables that are less visible by traditional logistic regression. CART analysis was performed using the HelixTree (Golden Helix) software. All *P* values were two sided with a significance level of 0.05.

Results

The study included 803 bladder cancer patients and 803 controls matched to cases by age (±5 years) and sex. Although the study is frequency matched, but not one-to-one matched, the same number of cases and controls were included for genotyping. We restricted the analysis to Caucasians due to the small sample size of minorities

and the concern about population stratification. By study design, cases and controls were well matched in terms of age and sex (Table 1). As predicted, there was a significant difference between cases and controls by smoking status ($P < 0.001$) with a higher percentage of current smokers in cases and higher percentage of never smokers in controls ($P < 0.001$). Among smokers, cases reported a higher pack-years of smoking than controls (38.0 versus 22.5, $P < 0.001$). Cases also had higher daily energy intake than controls (2,218.95 Kcal/d versus 1,991.39 Kcal/d, $P < 0.001$). Further, compared with controls, cases were less likely to take part in physical activity ($P < 0.001$). For example, the percentage of subjects that took part in intensive activities was 33.33% of controls but only 16.56% of cases ($P < 0.001$). No significant difference was observed in mean BMI between the two groups [27.31 kg/m^2 (cases) versus 27.51 kg/m^2 (controls), $P = 0.385$].

Among the 222 SNPs examined, 213 SNPs are in agreement with HWE. In the main effect of single SNP analysis, after adjusting for age, sex, tobacco smoking, BMI, energy intake, and physical activity, 28 of 222 SNPs had significant associations with bladder cancer risk (note that 27 of the 28 SNPs are in agreement with HWE. One SNP rs2271612 had a HWE test P value of 0.03). These SNPs were located in six genes in the mTOR pathway, including *AKT3*, *RHEB*, *RPS6KA5*, *IRS2*, *TSC2*, and *RAPTOR*. Because multiple SNPs within the *RAPTOR* gene showed significant main effect, only one SNP with the smallest P value within the same haplotype block was selected, resulting in 17 SNPs with significant main effects (Table 2).

SNPs rs7211818 in the *RAPTOR* gene remained significant after adjustment for multiple comparisons at the 10% level. The homozygous variant GG genotype was associated to an increased risk of bladder cancer, with ORs (95% CIs) of 2.16 (1.35-3.47). To assess the cumulative effects of all SNPs in the pathway, we further categorized the subjects into three risk groups according to the number of unfavorable genotypes: low-risk group (none to four unfavorable genotypes), medium-risk group (five to six unfavorable genotypes), and high-risk group (seven or more unfavorable genotypes). Unfavorable genotypes were defined by referring the ORs of genotypes showing a significant association ($P < 0.05$) in single SNP analysis in the best fitting model (as shown in Table 2). Compared with subjects in the low-risk group, the risks for the medium-risk group and the high-risk group were significantly elevated, with the ORs of 1.50 (95% CI, 1.17-1.93; $P < 0.001$) and 2.42 (95% CI, 1.81-3.23; $P < 0.001$), respectively.

We next assessed the main effects of energy balance–related variables: energy intake, physical activity, and BMI. Compared with subjects with the lowest tertile of energy intake (<1,738 Kcal/d), the OR for medium energy intake (1,738-2,312 Kcal/d) was 0.95 (95% CI, 0.72-1.25) and increased to 1.60 (95% CI, 1.23-2.09; $P = 0.001$) for the highest tertile of energy intake (≥2,313 Kcal/d; Table 3). Further, the increased risk that associated with higher energy intake was only significant in former (OR, 1.60; 95% CI, 1.11-2.32; $P = 0.012$) and current smokers (OR, 2.31; 95% CI, 1.02-5.22; $P = 0.044$) but not in never smokers. The interaction between smoking status and energy intake

Table 5. The association between energy balance index and bladder cancer stratified by the number of unfavorable genotypes

Energy balance index*	0-4 unfavorable genotypes			5-6 unfavorable genotypes			≥7 unfavorable genotypes		
	Case/control	OR (95% CI)†,‡	*P*	Case/control	OR (95% CI)†,‡	*P*	Case/control	OR (95% CI)†,‡	*P*
Q1	10/37	Reference		14/15	3.56 (1.27-10)	0.016	8/18	1.98 (0.65-5.98)	0.227
Q2	47/80	1.93 (0.87-4.3)	0.108	38/66	1.96 (0.86-4.44)	0.108	40/31	5.06 (2.15-11.9)	<0.001
Q3	86/123	2.49 (1.16-5.36)	0.019	72/104	2.84 (1.31-6.16)	0.008	67/54	4.85 (2.18-10.79)	<0.001
Q4	71/82	2.83 (1.3-6.18)	0.009	83/51	5.67 (2.56-12.57)	<0.001	55/27	7.19 (3.05-16.91)	<0.001
Q5	43/34	4.26 (1.82-9.98)	0.001	66/21	10.59 (4.41-25.39)	<0.001	36/5	21.93 (6.7-71.77)	<0.001

*Energy balance index is calculated by the combination of energy intake and physical activity. Q1, low energy intake + intensive physical activity; Q2, low energy intake + medium physical activity or medium energy intake + intensive physical activity; Q3, low energy intake + low physical activity or medium energy intake + medium physical activity or high energy intake + intensive physical activity; Q4, medium energy intake + low physical activity or high energy intake + medium physical activity; Q5, high energy intake + low physical activity.

†Adjusting for age, sex, tobacco smoking, and BMI where appropriate.

‡Refer to individuals carrying 0 to 4 unfavorable genotypes and with lowest energy balance index (Q1).

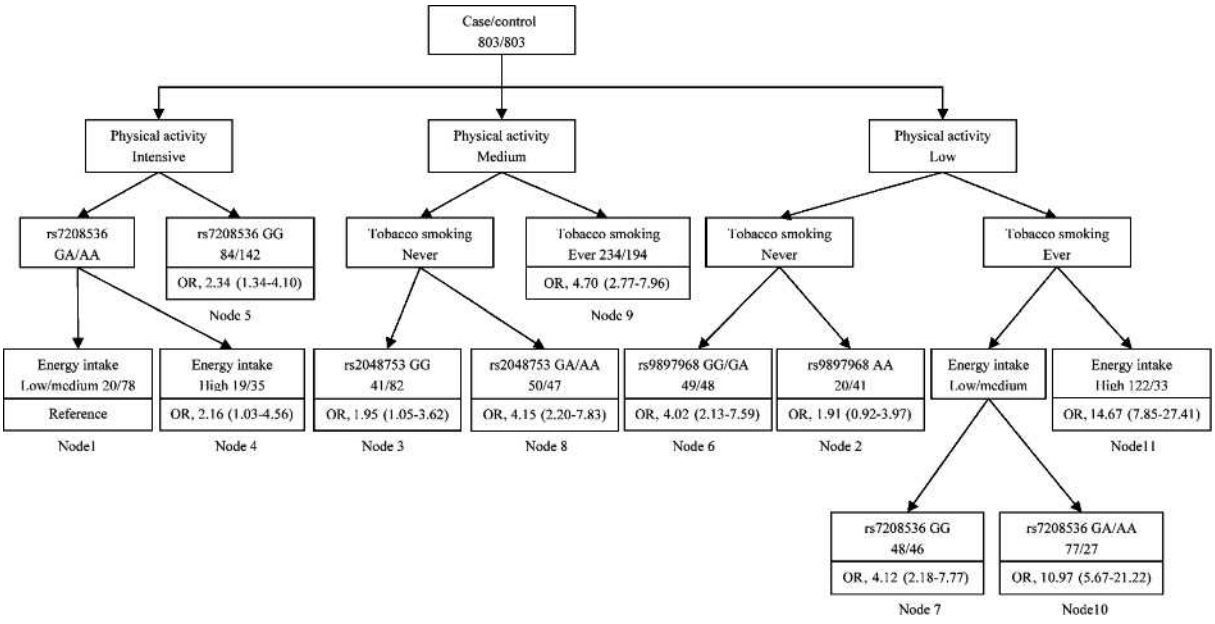

Fig. 2. CART analysis of lifestyle factors and genetic polymorphisms in the mTOR pathway in the risk of bladder cancer. ORs and 95% CIs (in parenthesis) are presented underneath each terminal node box.

was statistically significant ($P_{interaction}$ = 0.033). For physical activity, compared with subjects with intensive physical activity (MET ≥25), subjects with medium physical activity (MET ≥9 but <25) was at 1.87-fold (95% CI, 1.42-2.47; P < 0.001) increased risk and the risk further increased to 2.82-fold (95% CI, 2.10-3.79; P < 0.001) among subjects with low physical activity (MET <9; Table 3). When stratified by smoking status, significantly increased risk of low physical activity was observed in all subjects regardless of smoking status (Table 3). For example, the ORs for low physical activity was 2.17 (95% CI, 1.32-3.57; P = 0.002), 2.87 (95% CI, 1.89-4.35; P < 0.001), and 6.33 (95% CI, 2.68-14.97; P < 0.001) in never, former, and current smokers, respectively. However, no association was observed for BMI with the ORs of overweight and obese subjects being 0.95 (95% CI, 0.73-1.23) and 0.79 (95% CI, 0.59-1.07), respectively, compared with the normal BMI category (Table 3). No association was observed when the analysis was stratified by smoking status (Table 3).

To explore how genotypes in the mTOR pathway may modify the association between energy balance–related

Table 6. CART terminal nodes and risk for bladder cancer

Node*	Case/control	Case rate (%)	OR (95% CI)†	*P*
Node 1	20/78	20.41	1	
Node 2	20/41	32.79	1.91 (0.92-3.97)	0.082
Node 3	41/82	33.33	1.95 (1.05-3.62)	0.034
Node 4	19/35	35.19	2.16 (1.03-4.56)	0.042
Node 5	84/142	37.17	2.34 (1.34-4.10)	0.003
Node 6	49/48	50.52	4.02 (2.13-7.59)	<0.001
Node 7	49/46	51.58	4.12 (2.18-7.77)	<0.001
Node 8	50/47	51.55	4.15 (2.20-7.83)	<0.001
Node 9	234/194	54.67	4.70 (2.77-7.96)	<0.001
Node 10	76/27	73.79	10.97 (5.67-21.22)	<0.001
Node 11	122/33	78.71	14.67 (7.85-27.41)	<0.001

*Refer to the CART analysis (Fig. 2).
†Adjusting for age and sex.

variables and bladder cancer, we performed an analysis stratified by the number of unfavorable genotypes (Table 4). For energy intake, no significant association between energy intake and bladder cancer risk was observed among subjects carrying none to four unfavorable genotypes. However, among subjects carrying five to six unfavorable genotypes, those with the highest tertile of energy intake had a significant 1.66-fold (95% CI, 1.05-2.62; P = 0.031) higher risk than those with the lowest tertile. Among subjects carrying seven or more adverse genotypes, the risk among the highest tertile of energy intake was 2.11-fold (95% CI, 1.16-3.87; P = 0.015).

There was a 2.39-fold increased risk associated with the lowest tertile of physical activity among subjects carrying a low number of unfavorable genotypes (none to four unfavorable genotypes). However, among subjects carrying five to six unfavorable genotypes, those with the lowest tertile of physical activity experienced a 3.72-fold increased risk (95% CI, 2.22-6.23; P < 0.001). Among subjects carrying seven or more unfavorable genotypes, the risk associated with low physical activity was 3.45-fold (95% CI, 1.76-6.76; P < 0.001). BMI was not associated with bladder cancer risk among subjects regardless of the number of unfavorable genotypes, although a slightly nonsignificant inverse association was observed among subjects carrying seven or more unfavorable genotypes.

We next assessed the joint effects of energy balance–related variables and genotypes. Because physical activity and energy intake, but not BMI, was each associated with bladder cancer risk, we first assessed the joint effects of physical activity and energy intake (Fig. 1). Compared with the reference group, i.e., subjects with the lowest tertile of energy intake and highest intensive of physical activity, significant increased risks were observed in several subgroups (Fig. 1) with the highest risk observed among subjects with highest energy intake and lowest physical activity (OR, 4.37; 95% CI, 2.59-7.37; P < 0.001).

The joint effects of energy intake, physical activity, and mTOR pathway genes were shown in Table 5. In this analysis, we developed an energy balance index to collapse the physical activity and energy intake combinations (see Table 5 footnotes for the definition) and then analyzed the integrated index with the number of unfavorable genotypes. As shown in Table 5, the reference group was subjects with low energy intake, intensive physical activity, and who carry a low number of unfavorable genotypes (none to four unfavorable genotypes). Compared with the reference group, the highest risk was observed in subjects located in the worst energy balance category: subjects with high energy intake, do low physical activity, and carry 7 or more unfavorable genotypes. The risk was 21.93-fold (OR, 21.93; 95% CI, 6.70-71.77; P < 0.001; Table 5).

We applied CART analysis to explore the higher order gene-gene and gene-environment interactions. As shown in Fig. 2, three life-style factors (tobacco smoking, physical activity, and energy intake) and three SNPs (rs7208536, rs9897968, and rs2048753) were identified as the defining variables in the tree model (Fig. 2). The final tree structure contained 11 terminal nodes, representing a range of low- to high-risk subgroups as defined by different gene-gene, gene-environment combinations. The initial split of the root node was physical activity, indicating the importance of energy expenditure for risk of bladder cancer. To calculate ORs as defined by the terminal nodes, we defined node 1 as the reference group, representing individuals that had high physical activity, low/medium energy intake, and the rs7208536 GA/AA genotype. The ORs of terminal nodes ranged from 1.91 to 14.67 (Table 6). The highest risk group was individuals in terminal node 11 (ever smokers with low physical activity and high energy intake). Compared with the reference group, the risk for bladder cancer in this group has 14.67-fold increase (OR, 14.67; 95% CI, 7.85-27.41).

Discussion

This is the first study to report the joint effects of energy balance components and genetic modification effects from the mTOR pathway genes in bladder cancer risk. We evaluated the association between energy intake, physical activity, obesity, and the risk of bladder cancer. We also assessed whether genetic polymorphisms in mTOR pathway–related genes may interact with energy balance to modify bladder cancer risk. We found that both high caloric intake and low physical activity conferred increased risk of bladder cancer and that this risk may be modified by polymorphisms in the mTOR pathway genes. Specifically, the risk associated with high energy intake and low physical activity was most evident among subjects carrying a high number of unfavorable genotypes in the mTOR pathway. Moreover, our results strongly suggested that physical activity, energy intake, and genetic variants in the mTOR pathway jointly influenced the risk: subjects in the worst energy balance category (high energy intake, low physical activity, and carrying 7 or more unfavorable genotype) were at a >20-fold increased risk compared with subjects within the most favorable energy balance category (low energy intake, intensive physical activity, low number of unfavorable genotypes).

In the current study, we observed an increased bladder cancer risk among those with the high daily energy intake. Little data exist on the associations between energy intake and bladder cancer risk, except for a null association reported in the Health Professionals Follow-up Study (20). However, our result is consistent with findings from other cancer sites including kidney (49), breast (50), and rectum (51). In a large population-based case-control study (49), high calorie intake was associated with a significantly 1.30-fold increased risk of renal cancer. In a large prospective study of >38,000 women (50), a significantly increased 1.25-fold risk of breast cancer was reported with

high energy intake. Our results further revealed that the increased risk from high energy intake was only observed in smokers and that there was a significant interaction between energy intake and smoking status.

Energy balance reflects the interplay between diet, physical activity, and genetic background (37, 52). Positive energy balance due to excessive caloric intake and/or decreased energy expenditure leads to obesity and, consequently, elevated levels of bioactive insulin-like growth factor-I, insulin adipokines, and proinflammatory cytokines, which promote a carcinogenesis process (37). Animal studies suggest that restricting energy intake delays disease onset, including cancer (53, 54), and increases life expectancy (55). Energy restriction affects adrenal and insulin metabolism, as well as various gene expressions (56). Restriction of calorie intake by 10% to 40% has been shown to decrease cell proliferation, increasing apoptosis through antiangiogenesis processes (4).

Although the potential anticancer effect of calorie restriction is clear, it is not generally considered to be an independent feasible strategy for cancer prevention in humans because calorie restriction is difficult to maintain over time. Thus, physical activity, which helps increase energy expenditure, may be an alternative way to maintain energy balance. Compelling evidence has suggested that physical activity may enhance carcinogen detoxification; promote DNA repair process; alter cell proliferation, apoptosis, and differentiation; decrease inflammation; and enhance immune function (57). Consistent with the above anticancer roles of physical activity, we observed a strong inverse association between physical activity and bladder cancer risk and that the association was not modified by smoking status. In published literature, few studies specifically examined the relation between physical activity and bladder cancer, and when reported, the results were generally negative (14, 15, 20). In a large prospective cohort study of U.S. men and women (14), intensive physical activity had a borderline significant inverse association with bladder cancer with a relative risk (RR) of 0.87 (95% CI, 0.74-1.02) but the association was observed in former smokers only. In another large cohort study using data collected in the Health Professionals' Follow-up Study and the Nurses' Health Study (20), total recreational physical activity was not associated with bladder cancer with a RR of 0.97 (95% CI, 0.77-1.24). Lack of an association was also observed in some early studies with small number of bladder cancer cases (16–18, 58). However, in the Iowa Women's Health Study of postmenopausal women (15), an inverse association between regular physical activity and bladder cancer was borderline significant (RR = 0.66; 95% CI, 0.43-1.01). In contrast, an increased risk (RR = 2.06; 95% CI, 1.08-3.95) in bladder cancer was reported in a prospective study in middle-aged men in the United Kingdom (13). Physical activity is a complex behavior and the quantification of physical activity is complicated because it involves not only measures of frequency, intensity, and duration, but also multiple categories of physical activity (leisure time, occupation, household, transportation, etc.). Thus, physical activity is not precisely measured in most epidemiologic studies. Other possible explanations to the inconsistent results include the different study design, lack of objective measure of physical activity, lack of control for confounding, or chance findings due to small sample size in some studies.

One limitation of the abovementioned studies is that genetic factors were not considered in risk assessment. In light of genetic predisposition to energy balance and cancer risk and because the mTOR pathway is directly engaged in energy balance, we stratified the analysis of energy balance by genetic variants in the mTOR pathway to further elucidate the association between energy balance and bladder cancer. Interestingly, we found that the risk associated with high energy intake and low physical activity was only evident among subjects carrying a high number of unfavorable genotypes in the mTOR pathway.

Energy balance could modulate cellular signaling through cell surface receptors, affecting the activation or deactivation of multiple downstream genes within the mTOR pathway. The PI3K-AKT-mTOR signaling pathway is commonly activated in cancers (59). The signal is initiated by growth factors, nutrients, mutagens, and hormones that bind receptor tyrosine kinases, which then activate PI3Ks, resulting in a kinase cascade through AKT and mTOR, generating cell survival, growth, differentiation, cell cycle control, and angiogenesis signals (60), molecular processes implicated in cancers (39). As energy balance could affect the activation or deactivation of multiple downstream genes within the mTOR pathway and as the full activation of the mTOR pathway requires signals from both nutrients and growth factors, it is important to evaluate the role of mTOR pathway genes and cancer risk in the context of energy balance status (61). Although the mTOR pathway has been implicated in energy balance regulation, current evidence about the interaction between the mTOR pathway and energy balance in cancer risk from epidemiologic studies is scarce. To our knowledge, our current study is the first to explore the joint effects of genetic polymorphisms in the mTOR pathway and energy balance in bladder cancer risk. One important observation is that increased risk of bladder cancer associated with high energy intake and low physical activity was only observed in subjects carrying a high number of adverse genotypes, supporting strong modification effects from genetic variants in the energy balance–bladder cancer association. Moreover, when physical activity, energy intake, and genetic variants were analyzed jointly, the study population was clearly stratified into a range of low-to high-risk subgroups with subjects located in the worst energy balance category (high energy intake, low physical activity, and carrying 10 or more unfavorable genotype) experiencing the highest risk of bladder cancer. Our results strongly support the joint effects of physical activity, energy intake, and genetic variants in the mTOR pathway in bladder cancer susceptibility.

In our current study, we observed no association between obesity and bladder cancer risk in overall analysis and in stratified analysis by smoking status. The association between BMI and bladder cancer has been inconsistent in the literature. The majority of prospective cohort studies reported modest positive association (14, 20, 22–27). Some of these associations reached statistical significance whereas others did not. No association between baseline BMI and bladder cancer was observed in a large cohort study of health professionals (20). However, a significant positive association was found (RR = 1.33; 95% CI, 1.01-1.76) after excluding cases diagnosed within first 4 years of follow-up. A recent cohort study, the National Institute of Health-American Association of Retired Persons (NIH-AARP) Diet and Health Study (14), observed up to 28% increased risk of bladder cancer associated with obesity in a significant dose-response pattern. In contrast, two other cohort studies reported nonsignificant inverse associations between BMI and bladder cancer (15, 29). The reasons for the inverse association is unknown and is possibly due to the residual confounding from smoking. Compared with cohort studies, the results from case-control studies are more divergent with reporting positive, inverse, or null associations (31–34). The change in weight due to cancer diagnosis in cases could bias the results in case-control studies. The reference periods from which BMI were calculated are not consistent in previous studies and some studies did not report the reference period. Compared with prospective cohort studies, the bias in BMI due to the retrospective design may have led to the more inconsistent results in case-control studies. The association between obesity and bladder cancer warrants further study. Moreover, the inconsistencies could be attributed genetic risk factors yet to be identified.

We further explored the association between BMI and bladder cancer using weight 5 years before cancer diagnosis, weight 1 year ago before cancer diagnosis, and current weight at cancer diagnosis. Inverse associations were found in all three scenarios. However, the magnitude of the association was different. The OR for the obese subjects was 0.79 (95% CI, 0.59-1.07) if BMI 5 years ago was used. The ORs for BMI 1 year ago and current BMI were 0.74 (95% CI, 0.55-0.99) and 0.63 (95% CI, 0.47-0.85), respectively. We believe that the significant inverse association with current weight might largely be a result of weight loss due to bladder cancer diagnosis. To minimize such bias, we chose to use weight 5 years before cancer diagnosis to calculate BMI. Because data based on recall of diet and physical activity 5 years ago may not be accurate, in our study, we only collected physical activity data and dietary intake data in reference to 1 year before cancer diagnosis. Taken together, although not ideal, the use of weight 5 years ago and calorie intake and physical activity 1 year ago may be a reasonable choice in this study.

Our data suggest that the high calorie intake and low physical activity of bladder cancer cases may not result in high BMI. The reason is unknown, but it should be noted that energy balance is maintained by a complex system involving multiple interactive pathways and that the three simple variables (calorie intake, physical activity, and BMI) are not sufficient to describe the overall energy balance status. Other dietary factors, energy expenditure variables, and/or genetic factors not measured in this study may have contributed to the discrepancy and warrant further investigation.

In this study, besides the traditional logistic regression model, we also adopted a nonparametric CART analysis to explore the potential gene-gene and gene-environment interactions. The terminal nodes as defined by the combination of specific SNPs and life-style factors reflect risk subgroups from the interactions between genetic and environmental factors. The grouping of specific genotypes in the nodes of CART analysis may not be consistent with the results from single SNP analysis using logistical regression modeling, which did not take account of the higher order interactions between variables. The complementary application of both techniques has proved to be a promising approach to perform the task of analyzing and interpreting multiple marker studies.

Several methodologic issues should be discussed. One limitation in case-control studies is recall bias, in which healthy controls are more likely to recall "healthy lifestyle" than cancer patients (e.g., frequent physical activity, healthy diet pattern with less fat intake, etc.), leading to biased estimates of relative risk. Although the analyses presented have well controlled for confounders including age, sex, and tobacco smoking, other bladder cancer risk factors may have confounded the observed associations. Moreover, the case-control study design may subject to selection bias, in which the energy intake and physical activity of participants and nonparticipants may differ. Thus, prospective cohort studies are needed to better unveil the causal relationship between physical activity, energy intake, and cancer.

In conclusion, our results strongly support that both high caloric intake and low physical activity conferred increased bladder cancer risk and that the risk may be modified by genetic polymorphisms of mTOR pathway genes. The joint effects of physical activity, energy intake, and genetic variants in the mTOR pathway strongly suggest that bladder cancer susceptibility could be modified by energy balance status as defined by energy intake, physical activity, and genetic variants in the mTOR pathway genes.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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