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Folate metabolism genes, vegetable intake and renal cancer risk in central Europe

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In a multicenter case-control study of renal cell carcinoma (RCC) conducted in central and eastern Europe, we reported a strong inverse association with high vegetable intake and RCC risk. The odds ratio (OR) for high compared to the lowest tertile of vegetable intake was OR = 0.67; (95% confidence interval (CI): 0.53–0.83; *p*-trend < 0.001). We hypothesized that variation in key folate metabolism genes may modify this association. Common variation in 5 folate metabolism genes (*CBS: Ex9+33C > T* (rs234706), *Ex13 +41C > T* (rs1801181), *Ex18 −391 G > A* (rs12613); *MTHFR:* A222V *Ex5+79C > T* (rs1801133), *Ex8−62A > C* (rs1801131); *MTR: Ex26 20A > G* (rs1805087), *MTRR: Ex5+136 T > C* (rs161870), and *TYMS:IVS2−405 C > T* (rs502396), *Ex8+157 C > T* (rs699517), *Ex8+227 A > G* (rs2790)) were analyzed among 1,097 RCC cases and 1,555 controls genotyped in this study. Having at least 1 variant T allele of *MTHFR* A222V was associated with higher RCC risk compared to those with 2 common (CC) alleles (OR = 1.44; 95% CI: 1.17–1.77; *p* = 0.001). After stratification by tertile of vegetable intake, the higher risk associated with the variant genotype was only observed in the low and medium tertiles (*p*-trend = 0.001), but not among those in the highest tertile (*p*-interaction = 0.22). The association remained robust after calculation of the false discovery rate (FDR = 0.05). Of the 3 *TYMS* SNPs examined, only the *TYMS* IVS2 −405 C (rs502396) variant was associated with a significantly lower risk compared to the common genotype (OR = 0.73; 95% CI: 0.57–0.93). Vegetable intake modified the association between all 3 *TYMS* SNPs and RCC risk (*p*-interaction < 0.04 for all). In summary, these findings suggest that common variation in *MTHFR* and *TYMS* genes may be associated with RCC risk, particularly when vegetable intake is low.

Key words: kidney cancer; folate metabolism; 1-C metabolism; TYMS; MTHFR; genetic susceptibility; diet; vegetable intake

Epidemiological studies have reported associations between low folate intake and increased cancer risk.[1–5] Folate is involved in DNA methylation and synthesis and deficiency has been hypothesized to increase risk of cancer. Although the epidemiologic evidence strongly supports an association between low folate and higher risk of colon cancer, associations with other cancers, and notably kidney cancer, are less clear. An International Agency for Research on Cancer (IARC) working group recently determined that a protective effect of high vegetable consumption on renal cell cancer (RCC) was possible but not established because of several studies showing no association and the potential for confounding from other risk factors.[6] Folate, found in vegetables, legumes and fruits is necessary for the *de novo* synthesis of thymine. By decreasing the availability of N^5,N^{10}-methylenetetrahydrofolate, low folate leads to the misincorporation of uracil into DNA and subsequently single strand DNA breaks.

Efficient functioning of the folate (one-carbon) metabolism pathway is dependent upon several important enzymes such as methylene tetrahydrofolate reductase (MTHFR) and thymidylate synthase (TYMS), dietary intake of folate, B vitamins as cofactors, and methionine, an essential amino acid that is derived from protein intake. Among several genetic polymorphisms in this pathway characterized thus far, 2 closely linked SNPs in the *MTHFR* gene, A222V (codon 677C > T) and E429A (codon 1298A > C) which result in lower functional enzyme activity have received the most attention.[7–9] In relation to cancer, common variation in other genes in this pathway have been examined less frequently but may also modify cancer risk.[8,10–13] Like *MTHFR*, alterations in other genes can result in modifications in enzyme activity, changes in the capacity to remethyate homocysteine to methionine, alteration of plasma homocysteine levels and global DNA methylation levels.[14]

Recently, in a renal cancer case-control study conducted in Central and Eastern Europe, we reported a strong inverse association with high vegetable intake.[15] The inverse relationships were significant for total vegetable, cruciferous vegetable and yellow-orange vegetable consumption. Since many vegetables are also sources of folate and vitamin B6, we sought to determine whether common variation in 5 folate pathway genes (*CBS, MTHFR, MTR, MTRR, TYMS*) modified RCC risk among 1,097 cases and 1,555 controls enrolled in the Central and Eastern European Renal Cancer Case-Control study. We also investigated interactions with total vegetable consumption frequency as an estimation of nutrient intake relevant to this metabolic pathway.

Material and methods

Methods

From August 1999 through January 2003, we conducted a hospital-based case-control study of RCC in 7 centers across Central and Eastern Europe (Moscow, Russia; Bucharest, Romania; Lodz, Poland; and Prague, Olomouc, Ceske Budejovice, and Brno,

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TABLE I – GENES AND SINGLE NUCLEOTIDE POLYMORPHISMS IN THE FOLATE METABOLISM PATHWAY ANALYSED IN THE CENTRAL AND EASTERN EUROPEAN RCC CASE-CONTROL STUDY

Name of gene	Function in the 1-carbon metabolism pathway	Chromosome location	Nucleic acid change	Amino acid change	dbSNP ID
Cystathione beta-synthase	Catalyzes the first step of trans sulfuration pathway of homocysteine to cystathionine. Deficiency of this enzyme is known to cause homocysteineuria, -emia.	21q22.3	Ex9+33C > T Ex13+41C > T Ex18−391G > A	Y233Y A360A	rs234706 rs1801181 rs12613
5,10 methylene tetrahydrofolate reductase	Catalyzes the conversion of 5,10 methylenetetrahydrofolate, a co-substrate for homocysteine remethylation to methionine. Can be mutated in cases of homocysteineuria.	1p36.3	Ex5+79C > T Ex8−62A > C	A222V E429A	rs1801133 rs1801131
5-methyltetrahydrofolate-homocysteine methyltransferase	Also known as cobalmin-dependent methionine synthase (MS). Catalyzes the final step in methionine biosynthesis. Mutations cause methylcobalamin deficiency complementation group G.	1q43	Ex2620A > G	D919G	rs1805087
5-methyltetrahydrofolate-homocysteine methyltransferase reductase	Regenerates functional MTR (MS) via reductive methylation. Mutations cause disorders of folate cobalamin metabolism.	5p15.2-3	Ex5+136T > C	L206L	rs161870
Thymidylate Synthetase	Uses methylene THF as a cofactor to maintain dTMP pool critical for DNA replication and repair.	18p11.32	IVS2−405T > C Ex8+157C > T Ex8+227A > G	 3′UTR 3′UTR	rs502396 rs699517 rs2790

Czech Republic). A total of 1,097 patients newly diagnosed with histologically confirmed RCC (IDC-O-2 codes C64) between the ages of 20 and 79 years were recruited for this study. Trained medical staff reviewed hospital records to extract relevant diagnostic information, including date and method of diagnosis, histological classification, tumor location, stage and grade. Eligible controls ($N = 1,555$) were patients admitted to the same hospital as cases for conditions unrelated to smoking or genitourinary disorders (except for benign prostatic hyperplasia) and were frequency-matched to cases on age, sex, and study center. No disease was included among more than 20% of the control group. For controls, patient hospitalizations included the following categories of diseases/disorders: infectious (1.1%), hematologic (3.2%), endocrine (2.0%), psychiatric (1.4%), neurologic (11.2%), ophthalmologic or otologic (14.5%), cardiovascular (9.6%), pulmonary (3.9%), gastrointestinal (18.7%), dermatologic (2.8%), orthopedic or rheumatologic (8.9%), benign prostatic hyperplasia (3.8%), obstetric or perinatal (0.1%), injury or poisoning (3.0%), and other (15.9%). Both cases and controls had to be residents of the study areas for at least 1 year at the time of recruitment. The center-specific response rates for study participation ranged from 90.0 to 98.6% for cases and from 90.3 to 96.1% for controls. All subjects and their physicians provided written informed consent. This study was approved by the institutional review boards of all participating centers.

Interviews were conducted in person by trained personnel using standardized lifestyle and food frequency questionnaires as previously described. Details of the assessment of dietary intake can be found elsewhere.[15] Briefly, the dietary component of the questionnaire comprised 23 food items, which were selected by consensus by the study investigators during the planning stage of the study and further validated during the pilot study by asking participants to name common food items that were not already specified. Frequency of consumption and score [0–5] were assessed for each item was as follows: never [0], once per month [1], <once per week [2], 1–2 times per week [3], 3–5 times per week [4] and daily [5]. A standardized translated questionnaire was used in each of the study centers that was first translated from a common English version, and back-translated into English to ensure the validity of the initial translation. The questionnaire was repeated for 2 different time periods: the year prior to interview, and prior to political and market changes in 1989 (1991 in Russia). A life-time weighted average of vegetable intake for the 2 time periods was calculated by multiplying the score for each specific vegetable score for each time period by the number of years alive during the time period, summing the time period scores and dividing by the subject's age. Sensitivity analyses were conducted and determined that associations between renal cell carcinoma and dietary consumption patterns did not differ before and after political and market changes in 1989 (1991 in Russia) in adjusted multivariate logistic regression models (results not shown). Score values that correspond to intake frequencies of related individual food items were summed to form food groups as previously described for all vegetables. Individual types of vegetables considered in the questionnaire included cabbage, broccoli, and Brussels sprouts, tomatoes, pumpkin, carrots, leafy greens such as spinach, and other fresh/preserved vegetables that were regularly consumed in the study region. Tertiles were calculated based on the consumption frequencies observed among controls. Smoking status was defined as status 2 years before the interview (never smoker, former smoker, current smoker).

Laboratory procedures

Blood and buffy coat samples were stored at −80°C and shipped to the NCI biorepository on dry ice. DNA was extracted using phenol-chloroform extraction. Genotyping was conducted at NCI's Core Genotyping Facility (CGF). Descriptions of SNPs can be found in Table I. Genes were selected based on their role in the one-carbon metabolism pathway. SNPs were selected from those available for genotyping at the NCI CGF that had a minor allele frequency > 0.05, based on evidence of functionality from previous reports, non-synonymous SNPs, or those located in the 3′ untranslated region (UTR) which contains regulator sequences and binding sites for other molecules that could alter the stability of the mRNA transcript of the gene.

Genotype assay methods can be found at: http://snp500cancer.nci.nih.gov/home.cfm.[16] Laboratory personnel were blinded to case-control status, samples were blinded and randomized on PCR plates to avoid any potential bias and duplicate genotyping performed for a randomly selected 5% of the total series for quality control. Call rates for cases and controls were similar and for all genes were greater than 99% except for *CBS* Ex13+41C > T (97%), *MTRR* Ex5 + 135T > C (96%), *TYMS* IVS2 − 405 T > C

TABLE II – FREQUENCY DISTRIBUTION OF DEMOGRAPHIC VARIABLES AMONG SUBJECTS GENOTYPED IN THE CENTRAL AND EASTERN EUROPEAN RENAL CANCER STUDY

	Cases		Control		*p*-value[1]
	N	%	*N*	%	
Total subjects genotyped	925	42.6	1247	57.4	–
Center					
Bucharest, Romania	90	9.7	140	11.2	
Lodz, Poland	80	8.7	197	15.8	
Moscow, Russia	288	31.1	370	29.7	
Czech Republic[2]	467	50.5	709	43.3	<0.0001
Sex					
Male	550	59.5	807	64.7	
Female	375	40.5	440	35.5	0.01
Age at interview					
<45	90	9.7	122	9.8	
45–55	220	23.8	305	24.5	
55 < 65	281	30.4	382	30.6	
65 < 75	293	31.7	377	30.2	
75+	41	4.4	61	4.9	0.68
Smoking status[3]					
Never	433	46.9	507	40.7	
Former	205	22.2	311	24.9	
Current	285	30.9	428	34.3	0.01
Body mass index at interview					
<25	266	28.8	448	36.0	
25–<30	405	43.8	518	41.7	
30+	254	27.5	277	22.3	<0.001
Self-reported hypertension					
Yes	417	45.1	477	38.2	
No	507	54.9	770	61.8	0.001
Frequency of vegetable intake in tertiles[4]					
High[5]	236	26.2	410	33.8	
Medium	370	41.1	464	38.2	
Low[6]	294	32.7	340	28.0	<0.001

[1]*p*-value unadjusted, logistic regression used for all tests except for center, where a χ^2 test was used.–[2]Brno, Olomoue, Prague, Ceske Budejovice.–[3]Not significant after adjustment for sex, age and center.–[4]Tertiles based on distribution of vegetable intake frequency among controls.–[5]Using the high intake group as reference, OR(95% CI), medium intake group OR = 0.92;(0.75,1.15), low intake group OR = 0.67(0.53,0.83).–[6]Using the low intake group as reference, OR(95% CI), medium intake group OR = 1.45;(1.16,1.81), high intake group OR = 1.45(1.13,1.86).

(94%) and *TYMS* Ex8 +227A > G (96%). The prevalence of all genotypes among controls fell within the expected distributions of Hardy-Weinberg equilibrium ($p > 0.05$). Concordance rates for duplicates were 100% for all SNPs except *TYMS* Ex8 +227A > G (>99%).

Statistical analyses

For all genotype frequencies, heterogeneity between countries was tested among controls using the standard Q-test and significant differences were not observed. Polymorphism frequencies among controls were also compared to Caucasian controls in the SNP500 database.[16] Hardy–Weinberg equilibrium in controls was tested by the Chi-square goodness of fit test. Associations between SNPs and RCC risk were estimated using logistic regression after adjustment for (*i*) sex, age at interview and study center, and (*ii*) sex, age at interview and body mass index (BMI) as continuous variables, study center, self-reported hypertension (no/yes), and family history of cancer. Because the genotypes did not modify the relationships observed between BMI, hypertension, and RCC and because the inclusion of additional lifestyle variables in addition to sex, age, and center did not modify ORs by more than 10%, results controlling only for sex, age at interview and study center are presented. Both *MTHFR* polymorphisms were included in regression models of each other as they are in linkage disequilibrium, are both associated with enzyme activity, and because their inclusion modified ORs by ~10% from those estimated in a model that included only sex, age and center. To assess multiplicative interactions, comparison of regression models with and without interaction terms using a likelihood ratio test (LRT) was conducted. Haploview was used to assess linkage disequilibrium among SNPs from the same gene. Haplotypes were estimated for genes with more than one SNP using SAS/Genetics, and the haplotype pair with the highest posterior probability for each subject was included in a logistic regression model, adjusting for age, sex and center. The global score test implemented in HaploStats (version 1.2.1) was used to evaluate overall differences in the haplotype frequencies between cases and controls,[17] adjusting for the false discovery rate (FDR) procedure proposed by Benjamini and Hochberg.[18] All other analyses including logistic regression of haplotype variables were conducted in STATA 8.0 unless specified otherwise (STATA Corporation, College Station, TX).

Results

Genotyping data was available for 925 (84.2%) of cases and 1,247 (80.2%) of controls. Remaining subjects were not analyzed because they did not provide blood samples or DNA of sufficient quantity and quality required for analysis. Subjects without genotyping data were similar with respect to age and known RCC risk factors to those that were genotyped (data not shown). Most genotyped subjects included in the current analysis were from the Czech Republic followed by Moscow (Table II). Cases and controls were comparable by age distribution however there were more men with genotyping data among controls than cases ($p = 0.01$). Cases had higher BMI and self-reported hypertension than controls but their smoking habits were not different from controls after adjustment for sex, age and center. High consumption of all vegetables, including subgroups of cruciferous vegetables, and

TABLE III – POLYMORPHISMS IN FOLATE METABOLISM GENES AND RENAL CELL CANCER RISK

SNP	Case	Control	OR[1]	95% CI	*p*
CBS Y233Y (Ex9+33 C > T)					
CC	401	523	1.00		
CT	394	502	1.06	(0.87–1.20)	
TT	92	154	0.78	(0.59–1.05)	
Per allele			0.94	(0.83–1.07)	0.36
CT+TT	486	656	0.99	(0.83–1.19)	0.95
CBS A360 (Ex13+41 C > T)					
CC	289	382	1.00		
CT	408	543	1.04	(0.72–1.27)	
TT	133	199	0.91	(0.69–1.19)	
Per allele			0.97	(0.85–1.10)	0.62
CT+TT	541	642	1.00	(0.83–1.23)	0.97
CBS (Ex18−391G > A)					
GG	778	1048	1.00		
AG	101	127	1.11	(0.85–1.47)	
AA	4	0	NA		
Per allele			1.08	(0.81–1.42)	0.60
AG+GG	105	127	1.11	(0.85–1.47)	0.44
MTHFR A222V (Ex5+79 C > T)					
CC	355	556	1.00		
CT	370	419	1.45	(1.17–1.79)	
TT	93	113	1.40	(0.99–1.98)	
Per allele			1.27	(1.08–1.48)	0.003
CT+TT	463	532	1.44	(1.17–1.77)	0.001
MTHFR E428A (Ex8−62 A > C)					
AA	376	491	1.00		
AC	357	483	1.09	(0.88–1.35)	
CC	85	113	1.22	(0.86–1.73)	
Per allele			1.10	(0.94–1.29)	0.24
AC+CC	442	596	1.12	(0.90–1.34)	0.24
MTR D919G (Ex26 20A > G)					
AA	545	683	1.00		
AG	258	383	0.84	(0.69–1.02)	
GG	45	68	0.81	(0.59–1.21)	
Per allele			0.87	(0.75–1.01)	0.07
AG+GG	303	451	0.83	(0.69–1.01)	0.06
MTRR L206L (Ex5+136T > C)					
TT	667	923	1.00		
CT	129	153	1.14	(0.88–1.47)	
CC	8	11	0.91	(0.36–2.30)	
Per allele			1.09	(0.87–1.37)	0.45
CT+CC	137	164	1.12	(0.87–1.44)	0.38
TYMS (IVS2−405 C > T)					
TT	279	324	1.00		
CT	364	548	0.73	(0.57–0.93)	
CC	151	209	0.72	(0.50–1.04)	
Per allele			0.83	(0.69–1.00)	0.05
CT+CC	515	757	0.73	(0.57–0.93)	0.01
TYMS 3′UTR (Ex8+157 C > T)					
CC	420	549	1.00		
CT	377	500	1.05	(0.78–1.41)	
TT	68	100	1.10	(0.63–1.92)	
Per allele			1.05	(0.81–1.37)	0.71
CT+TT	445	600	1.05	(0.98–1.39)	0.76
TYMS 3′UTR (Ex8+227 A > G)					
AA	568	742	1.00		
AG	289	387	1.09	(0.82–1.44)	
GG	28	44	1.06	(0.55–2.04)	
Per allele			1.07	(0.82–1.38)	0.63
AG+GG	317	431	1.09	(0.82–1.44)	0.56

[1]All odds ratios (ORs) were estimated adjusting for sex, center and age. Since *MTHFR* A222V and E428 A are linked, therefore ORs were estimated including both polymorphisms in regression models.

yellow-orange vegetables were inversely associated with RCC as previously observed for the entire study population.[15]

Risk estimates for polymorphisms in folate metabolism genes are shown in Table III. The frequency of the *MTHFR* A222V and *TYMS* IVS2 −405 C variants differed significantly among cases and controls. Compared to subjects homozygous for the common CC allele of *MTHFR* A222V, RCC risk was higher among cases with one (OR = 1.45; 95% CI: 1.17–1.79) or 2 variant alleles (OR = 1.40; 95% CI: 0.99–1.98, *p*-trend = 0.001). The *TYMS* IVS2 −405 CT and CC (variant) genotypes were associated with lower risk compared to the common TT genotype (OR = 0.73; 95% CI: 0.57–0.93). The FDR values for these 2 findings were 0.05 and 0.3, respectively.

In Table IV, the individuals risk estimates of RCC as well as the joint effect model with vegetable intake are presented for the *MTHFR* and *TYMS* genotypes and haplotypes. Genetic variants in *CBS, MTR,* and the *MTRR* genes, were not associated with RCC risk alone or after stratification by vegetable intake. For the *MTHFR* gene, the elevated risk that was previously associated with the variant 222V allele overall was only apparent in the low vegetable intake group (CT: OR = 1.50; 95% CI: 1.04–2.17 and TT: OR = 1.94; 95% CI: 1.06–3.61; *p*-trend = 0.007). Significance was borderline for the middle tertile. Risk among those with the TT genotype decreased in an exposure-dependent manner across tertiles of vegetable intake (*p*-trend = 0.05). Modification of risk by genotype was no longer apparent when intake was high. The distribution of *MTHFR* haplotypes differed significantly between cases and controls (*p*-global = 0.005) and the *p*-value for the interaction with vegetable intake was 0.05.

The 3 *TYMS* variants, IVS2 −405 CC, 3′UTR (Ex8+157) TT, and 3′UTR (Ex8+227) GG, were associated with lower risk compared to the common genotypes when vegetable intake was low. After stratification by vegetable intake tertile, the highest risks for the 3 *TYMS* SNPs were observed among those with the most common variants when vegetable intake was low. The comparably higher risk observed with the common *TYMS* SNPs decreased significantly across exposure categories as vegetable intake increased ($p < 0.0001$ for all) and modification of risk by genotype was no longer apparent when intake was high. Multiplicative interactions were observed for all 3 *TYMS* SNPs and tertiles of vegetable intake (*p*-interaction = 0.04, 0.001, 0.03, respectively).

Haplotype analysis of the *CBS* gene revealed one rare haplotype (TTG-1% among cases and 2.1% among controls) was inversely associated with RCC risk when compared to the common haplotype as a referent (CCG) (OR = 0.55; 95% CI: 0.31–0.96), but the global test was not significant ($p = 0.25$). This association was observed independent of vegetable intake (*p*-interaction = 0.39).

Discussion

In this study, associations between RCC risk and common genetic variation in 10 SNPs in 5 folate metabolism genes were examined. Compared to individuals homozygous for the common *MTHFR A222V* C allele, risk was significantly elevated among subjects with at least one T variant at A222V allele. This finding was particularly robust as the false discovery rate value was low (FDR = 0.05) and the trend was significant using an additive model. The increase in RCC risk associated with the *MTHFR* A222V variant allele was significantly higher when vegetable intake was in the low or medium tertile. When haplotypes of *MTHFR* A222V and E428A were estimated using these data, a global test of haplotype distribution among case and controls was highly significant and an interaction with vegetable intake tertile was observed. Overall it appeared in the haplotype analysis that modification of RCC risk was primarily driven the by the C variant at A222V. We also observed that the *TYMS IVS2 −405 C* variant was associated with significantly lower risk among RCC cases when compared to the common TT homozygous genotype, but false discovery for this finding could not be ruled out (FDR = 0.3). When the 3 *TYMS* variants were examined in relation to vegetable intake, they were all associated with lower RCC risk compared to the common referent genotype, but mainly when vegetable intake was in the lowest tertile. Risk associated with genotype was also modified by vegetable intake and significant gene-nutrient interactions were observed. Among those with low vegetable intake, we also observed a significantly reduced risk with the CTG haplotype that included 3 variants, when compared to the most common haplotype.

TABLE IV – TYMS AND MTHFR POLYMORPHISMS, HAPLOTYPES, VEGETABLE INTAKE AND RENAL CELL CANCER RISK

Gene	All subjects Case/Control	All subjects OR[1]	All subjects 95% CI	Tertile of vegetable intake frequency[1] Low OR[2]	Low 95% CI	Medium OR[2]	Medium 95% CI	High OR[2]	High 95% CI	*p*-trend[3]	*p*-int[4]
MTHFR A222V (Ex5+79 C > T)											
CC		1.00		1.00		1.16	(0.83–1.62)	0.86	(0.59–1.26)	0.55	
CT		1.45	(1.17–1.79)	1.50	(1.04–2.17)	1.64	(1.16–2.34)	1.12	(0.81–1.75)	0.31	
TT		1.40	(0.99–1.98)	1.95	(1.06–3.61)	1.43	(0.85–2.43)	0.93	(0.49–1.75)	0.05	
p-trend[3]		0.001		0.007		0.05		0.59			0.22
MTHFR E428A (Ex8 −62 A > C)											
AA		1.00		1.00		1.03	(0.79–1.58)	0.80	(0.55–1.17)	0.39	
CA		1.09	(0.88–1.35)	1.03	(0.72–1.49)	1.12	(0.89–2.56)	0.85	(0.58–1.25)	0.34	
CC		1.22	(0.70–1.32)	1.19	(0.65–2.21)	1.51	(0.55–1.17)	0.65	(0.34–1.25)	0.12	
p-trend[3]		0.24		0.19		0.18		0.48			0.56
HAPLOTYPE-MTHFR A222V (Ex5+79 C > T): E428A (Ex8 −62 A > C)											
C-A	33.8/37.9	1.00		1.00		1.09	(0.85–1.38)	1.06	(0.82–1.39)		
C-C	32.1/32.5	1.10	(0.94–1.29)	1.14	(0.85–1.53)	1.26	(0.99–1.60)	0.96	(0.73–1.26)		
T-A	34.0/29.5	1.29	(1.1–1.51)	1.49	(1.12–1.99)	1.39	(1.09–1.77)	1.14	(0.87–1.49)		
T-C	0.0/0.001										
p-global[3]		0.005									0.05
TYMS (IVS2 −405 T > C)											
TT		1.00		1.00		0.84	(0.57–1.25)	0.42	(0.27–0.67)	<0.0001	
CT		0.73	(0.57–0.93)	0.47	(0.32–0.70)	0.66	(0.45–0.95)	0.54	(0.36–0.80)	0.28	
CC		0.72	(0.50–1.04)	0.67	(0.40–1.12)	0.60	(0.37–0.91)	0.49	(0.30–0.82)	0.35	
p-trend[3]		0.05		0.02		0.06		0.38			0.04
TYMS 3′UTR (Ex8+157 C > T)											
CC		1.00		1.00		0.89	(0.65–1.23)	0.47	(0.33–0.67)	0.0001	
CT		1.05	(0.78–1.41)	0.73	(0.48–0.93)	0.73	(0.53–1.01)	0.76	(0.53–1.08)	0.50	
TT		1.10	(0.63–1.92)	0.42	(0.21–0.85)	1.08	(0.63–1.85)	0.56	(0.30–1.04)	0.90	
p-trend[3]		0.71		0.01		0.73		0.06			0.001
TYMS 3′UTR (Ex8+227 A > G)											
AA		1.00		1.00		1.00	(0.76–1.32)	0.59	(0.43–0.80)	<0.001	
AG		1.09	(0.82–1.44)	0.75	(0.52–1.08)	0.82	(0.60–1.13)	0.92	(0.59–1.21)	0.28	
GG		1.06	(0.55–2.04)	0.43	(0.18–1.03)	1.91	(0.84–4.37)	0.23	(0.06–0.81)	0.35	
p-trend[3]		0.63		0.04		0.81		0.13			0.03
HAPLOTYPE-TYMS (IVS2 −405 T > C):3′UTR (Ex8+157 C > T): 3′UTR (Ex8+227 A > G)											
T-C-A	58.5/56.0	1.00		1.00		0.97	(0.82–1.15)	0.70	(0.58–0.85)		
T-T-A	0.4/0.4	1.11	(0.41–3.04)	1.18	(0.16–8.57)	1.58	(0.34–7.31)	0.43	(0.04–4.71)		
T-T-G	1.1/0.9	1.20	(0.64–2.24)	1.29	(0.38–4.37)	0.82	(0.33–2.05)	1.40	(0.38–5.13)		
C-C-A	12.2/13.8	0.88	(0.73–1.06)	0.90	(0.64–1.28)	0.70	(0.52–0.95)	0.68	(0.48–0.96)		
C-T-A	9.2/9.5	0.94	(0.76–1.18)	0.70	(0.45–1.09)	0.82	(0.57–1.16)	0.97	(0.66–1.41)		
C-T-G	18.6/19.4	0.93	(0.78–1.10)	0.65	(0.48–0.89)	0.89	(0.69–1.15)	0.84	(0.63–1.13)		
p-global[3]	0.63										0.15

[1]Tertiles based of frequency of vegetable intake among controls.–[2]adjusted for sex, center, and age, 1 MTHFR A222V and E428A are linked, therefore ORs were estimated.–[3]*p*-values for trend were calculated using logistic regression, adjusting for sex, age, and center.–[4]Interactions were tested using a likelihood ratio test and adjusting for sex, age, and center.

Sporadic RCC is a rare disease but it is increasing in incidence in North America and Europe. Generally, studies conducted to date have not been sufficiently powered to examine the role of common genetic variation and risk. One earlier study of cancer mortality in a population based cohort of elderly men identified an increased risk of kidney and bladder cancer (combined) among men with the *MTHFR* 222VV genotype (RR = 5.48; 95% CI: 1.67–18.0), although the exact number of cases of each cancer type was not reported.[19] Previous studies have generally not shown significant associations between the *MTHFR* 222VV genotype and bladder cancer risk,[19–22] suggesting that the excess risk reported by Heijmans *et al.*,[19] might have been driven by kidney cancer.

To our knowledge, the current study is the largest and most detailed evaluation of genetic variation within this pathway and kidney cancer risk conducted to date. Our findings support a role for both *MTHFR* and *TYMS* in modifying RCC risk. Furthermore, there is evidence that many of the SNPs we investigated are functional, or in linkage with variants that have demonstrated evidence of functionality in the literature.[7,8,13] *MTHFR* plays a central role in directing the folate pool toward the remethylation of homocysteine to methionine at the expense of purine and DNA synthesis. The *MTHFR A*222V is a functional polymorphism and the T variant results in a thermolabile enzyme with reduced activity that hinders nucleotide synthesis.[9] Reduced *MTHFR* activity lowers the availability of *S*-adenosyl methionine (SAM) required for DNA methylation. In the current study, this *MTHFR* variant was only associated with cancer risk when vegetable intake was low. This finding is in agreement with other studies that have demonstrated an increased role of genetic susceptibility when the availability of one-carbon nutrients is low.[23,24]

Although high folate intake has been associated with a reduced risk of most cancer sites studied, the effect of the *MTHFR A222V* variant appears to be different for different cancer sites. It is worthwhile to point out that the association of MTHFR222 variant observed in this study, is in the opposite direction for colorectal cancer.[5] The mechanism through which *MTHFR* variation may modify risk for different cancer sites is unclear. Nevertheless, this gene plays a central role in balancing DNA synthesis, methylation, and urocil misincorporation rates in tissues.

We also found the *TYMS* SNPs to modify the association between RCC risk and vegetable intake when analyzed individually or as a haplotype. Although we know little about the functionality of these particular SNPs, the *TYMS* Ex8+157 C > T SNP is linkage disequilibrium with the 1496delTTAAAG polymorphism that is also located in the 3′UTR region of this gene.[13] In this report the C allele at rs699517 is nearly 100% correlated with the *TYMS* 1496 insertion and the T allele with the deletion. This genotype has been associated with decreased mRNA stability and lower expression in tumor tissue than the wildtype polymorphism.[25,26] Another functional SNP in the *TYMS* gene (−96

5′UTR) was also in linkage disequilibrium with *TYMS* Ex8+157 C > T ($r^2 = 0.29$; $D' = 0.56$) and *TYMS* Ex8+227A > G ($r_2 = 0.11$; $D' = 0.59$) which were themselves highly correlated in this study ($r^2 = 0.35$; $D' = 0.99$).[16] A few other studies have found lower cancer risks associated with these functional *TYMS* variants.[13,27] *TYMS* is a key enzyme in the DNA synthesis pathway that is selectively blocked by certain chemotherapeutics such as 5-flurourocil. The gene is also highly active in RCC compared to normal tissue and activity increases significantly with tumor stage and grade.[27,28] These findings suggest that cancer cells harboring a variant *TYMS* gene encoding for an unstable protein might have lower proliferation rates compared to those with a normal gene. Alternatively, reduced thymidine synthesis may lead to the misincorporation of uracil into DNA.

The strengths of this study include high participation rates thus minimizing potential selection bias. The large sample size provided sufficient statistical power to detect relatively small associations between genotypes and risk, but the power to detect interactions was limited. Although we included a number of functional and non-synonymous SNPs in this analysis, we did not conduct a dense survey of tagged SNPs intended to capture a high proportion of haplotype diversity within the genes analyzed. It is possible that there may be additional genetic variants not captured in this analysis that may also be associated with cancer risk. While there are also some limitations with using hospital based case-control studies such as recall bias, hospital based study design can help improve response rates for intensive biological specimen collection and reduce risk of bias when assessing gene-environment interactions.[29] Lastly, the food frequency questionnaire used to determine vegetable intake frequency was not robust and we were not able to perform adjustment for energy intake nor were we able to assess quantities of vegetables consumed as our metric did not include such information. However, it was developed to properly capture dietary habits in the region under study and to assess dietary patterns before and after the political changes affecting eastern Europe in the late 1980s and early 1990s when dietary changes occurred as a result of entering the western market.

In summary, our study was the first to examine associations between folate metabolism, vegetable intake, and RCC risk in Eastern and Central Europe, a region with some of the highest rates of kidney cancer in the world. These data provide additional evidence for the role of common genetic variation in *MTHFR* and *TYMS* genes may significantly modify kidney cancer risk, particularly when vegetable intake is low.

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