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Associations between Cigarette Smoking, Hormone Therapy and Folate Intake with Incident Colorectal Cancer by TP53 Protein Expression Level in a Population-Based Cohort of Older Women

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Abstract

Cigarette smoking (CS), hormone therapy (HT) and folate intake (FI) are each thought to influence colorectal cancer (CRC) risk, but the underlying molecular mechanisms remain incompletely defined. The TP53 (p53) protein, encoded by the *TP53* tumor suppressor gene that is commonly mutated in CRC, can be readily assessed to differentiate biologically distinct CRC subtypes. In this prospective cohort study, we examined CS, HT, and FI -associated CRC risks by TP53 protein expression level among Iowa Women's Health Study (IWHS) participants. The IWHS recruited 41,836 randomly selected Iowa women, ages 55–69 years, with a valid driver's license at study entry in 1986. Self-reported exposure variables were assessed at baseline. Incident CRC cases were ascertained by annual linkage with the Iowa Cancer Registry. Archived, paraffin-embedded tissue specimens were collected and evaluated for TP53 protein expression by immunohistochemistry. Multivariate Cox regression models were fit to estimate relative risks (RRs) and 95% confidence intervals (CIs) for associations between CS, HT, or FI and TP53-defined CRC subtypes. Informative environmental exposure and protein expression data were available for 492 incident CRC cases: 222 (45.1%) TP53 negative, 72 (14.6%) TP53 low, and 198 (40.2%) TP53 high. Longer duration (> 5 years) of HT was inversely associated with TP53 high CRCs (RR = 0.50; 95% CI = 0.27–0.94). No other statistically significant associations were

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observed. These data support possible heterogeneous effects from HT on TP53-related pathways of colorectal carcinogenesis in older women.

INTRODUCTION

Colorectal cancer (CRC) represents the fourth most common incident and second most common fatal cancer in the United States, with estimates of 142,820 new cases and 50,830 attributable deaths in 2013 (1). Molecular heterogeneity in colorectal carcinogenesis is well established (2-4) and may have implications for targeted prevention, early detection, and/or treatment strategies. With respect to CRC risk assessment, our group and others have observed differential associations between common environmental exposures, including cigarette smoking (CS), hormone therapy (HT) and folate intake (FI), and incident CRCs defined by microsatellite instability (MSI), CpG island methylator phenotype (CIMP), *KRAS* mutation, or *BRAF* mutation status (5-10). However, to date, relatively fewer studies have examined subtype-specific CRC risks by TP53 expression levels (11-12).

Somatic mutations in the *TP53* tumor suppressor gene are reportedly found in 43% of all CRC cases (13). In normal tissue, TP53 protein accumulation is difficult to detect by immunohistochemistry (IHC), due to tight regulation and rapid degradation. However, in the presence of a *TP53* mutation, TP53 protein accumulates in the nucleus (although its function is disrupted). Thus, IHC quantification of TP53 protein expression level can be applied as a reasonable surrogate for tumor suppression function, as previously described (11,13).

In this current study we used baseline data and archived tumor tissue specimens from the prospective, population-based Iowa Women's Health Study (IWHS) to examine associations between CS, HT, and FI with TP53-defined CRC subtypes in older women.

MATERIALS AND METHODS

This study was reviewed and approved by the Institutional Review Boards for Human Research of the University of Iowa, University of Minnesota and Mayo Clinic Rochester.

Subjects

Recruitment and enrollment methods for the IWHS have been reported elsewhere (14). Briefly, a 16 page baseline questionnaire was completed and returned by 41,836 randomly selected women, ages 55-69 years, who resided in Iowa and held a valid driver's license at baseline in 1986. For the present study, exclusions (not mutually exclusive) were made based on: history of any malignancy other than skin cancer (n=3830) or follow-up less than one day (n=10). Additional exposure-specific exclusions were made based on incomplete exposure information (n=660 for CS and n=200 for HT); incomplete premenopausal or menopause status (for HT analyses only, n=569); or invalid dietary data (for FI analyses only, 30 missing dietary variables, < 600 calories or > 5000 calories per day, n=3096). Vital status and state of residence were determined by mailed follow-up surveys and through linkage to Iowa death certificate records.

Risk Factor Assessment

Comprehensive self-reported demographic, dietary, lifestyle, and medication data were collected during the baseline IWHS evaluation (1986). CS patterns, including smoking status (never, ever, former, current), smoking duration (years), average number of cigarettes smoked per day, and cumulative pack-years were collected. Dietary habits were assessed using a semi-quantitative food frequency questionnaire adapted from the 126-item instrument developed by Willett and colleagues (15). FI was computed by multiplying the

frequency response by the nutrient content of the specified portion sizes, with additional intake from supplement use included when indicated. Previous or current HT and duration of HT was also collected, as described previously(7). Potential confounding variables acquired from the baseline questionnaire included body mass index, waist-to-hip ratio, physical activity level, alcohol consumption, age at menarche, age at menopause, oral contraceptive use, history of diabetes mellitus and daily intake of total calories, fat, sucrose, red meat, calcium, vitamin E, and methionine.

Case Ascertainment

Incident CRC cases were identified through annual linkage with the Iowa Cancer Registry, which is a member of the National Cancer Institute's Surveillance, Epidemiology and End Results (SEER) program(16). CRC cases were identified using International Classification for Diseases in Oncology (ICD-O) codes of 18.0, 18.2-18.9, 19.9 and 20.9, with tumors located in the cecum, ascending colon, hepatic flexure, transverse colon and splenic flexure defined as proximal colon cancers and tumors located in the descending colon, sigmoid colon, rectosigmoid junction and rectum defined as distal colorectal cancers(17-18).

Tissue Selection and Processing

Beginning in 2006, archived, paraffin-embedded tissue specimens were requested from incident CRC cases diagnosed through December 31, 2002. In total, tissue specimens were retrieved from 732/1255 (58%) cases, which is similar to CRC tissue retrieval rates recently reported from the Health Professionals Follow-up Study (51%)(19) and the Nurses' Health Study (58%) (20). Subject demographics, exposure patterns, and tumor characteristics did not differ significantly between CRC cases with retrieved versus non-retrieved tissue specimens, as previously reported (5). All incident CRC diagnoses were confirmed by a single gastrointestinal pathologist. A total of 563/732 (77%) cases met criteria for the present study (i.e., confirmed first primary CRC with sufficient tissue for the planned laboratory analyses). Paraffin blocks were serially sectioned onto 5 or 10 um slides. The last slide was stained with hematoxylin and eosin (H&E) so that areas of neoplastic (defined as >50% dysplastic cells) and normal tissue could be defined and marked. From these marked slides, three tumor cores were taken from each block and placed into tissue microarray (TMA) blocks along with liver controls. The TMAs were produced by the Mayo Clinic Pathology Research Core lab using the Beecher ATA-27 automated array. From the TMAs, 5 um slides were cut for H&E or IHC staining.

Characterization of TP53 protein expression by immunohistochemistry

IHC for TP53 expression was performed by the Pathology Research Core (PRC) at the Mayo Clinic. Briefly, slides were deparaffinized and hydrated with distilled water, antigen retrieval was done by soaking slides in EDTA in 98-100° steamer for 30 minutes. A protein block was done (DAKO X0909) and primary antibody (TP53 Clone DO-7 DAKO M7001 at 1:200 dilution) was applied. The secondary HRP labeled antibody was applied (DAKO K4061) and chromagen DAB (DAKO K3468) was used and the sections were counterstained with hematoxylin. Each section or core was scored by a pathologist (TS) using a combination of the staining intensity (0-3) and percent of cells stained (0 = 0%, 1 = <1%, 2 = 1 to 10%, 3 = 10 to 30%, 4 = 31-67%, and 5 = > 67%). The two scores were added for a combined score (0-8) as reported by Harvey et al (21). Each case was classified as TP53 negative if the combined score was 0, TP53 low if the score was 1-5 and TP53 high for a score of 6-8 (representative examples shown in Figure 1). For each individual, the tumor core with the highest score was used for analysis.

TMAs have been used by others for IHC evaluation (11,22-23). To further assess the validity of this approach with our tissue set, we conducted a pilot study comparing the

scores obtained from whole sections versus TMA cores derived from the same FFPE blocks (n = 28). An 83% correlation was observed, supporting the use of the TMA for analysis in our study. We also compared the sensitivity and specificity of using IHC to detect cases with a TP53 mutation. Based on sequencing and IHC data for 34 cases, we found that TP53 high protein expression (Allred score ≥ 6) had 72 % sensitivity and 80% specificity for detecting TP53 mutations, similar to an earlier findings reported by Curtin et al(24).

Statistical Analysis

Follow-up was calculated as age at completion of the baseline survey until age at first CRC diagnosis, age at move from Iowa, or age at death. If none of these events occurred, a woman was assumed to be alive, cancer-free, and living in Iowa through December 31, 2002. Cox proportional hazard regression analysis was used to estimate relative risks (RRs) and 95% confidence intervals (CIs) for associations between exposures of interest and CRC subtypes defined by TP53 protein expression status (negative, low and high). For all subtype analyses, the outcome variable was incident CRC with the TP53 protein expression status of interest; all other CRC cases (including those with missing or unknown TP53 status) were considered censored observations at the date of diagnosis.

Three common environmental exposures (representing lifestyle habit, medication use, and dietary intake) were selected for analyses: CS, examined by overall status (never, ever, former, or current), average number of cigarettes smoked per day, and cumulative cigarette pack-years; HT, examined by overall status (never, ever, former, or current) and duration of use; and FI, examined by quartiles of consumption. Tests for trend were carried out for each exposure variable by ordering the categorized values from lowest to highest category and including the resulting variable as a linear term in the Cox regression model. Multivariable adjustments were applied as follows: all models adjusted for body mass index (BMI), waist-to-hip ratio (WHR), physical activity level, alcohol consumption and daily intake of total calories, fat, sucrose, red meat, calcium, vitamin E, and methionine. CS analyses also adjusted for HT and FI. HT analyses also adjusted for CS, FI, age at menarche, age at menopause, OC use, and history of DM. FI analyses additionally adjusted for CS, HT and history of DM.

We formally determined if risk ratios for the CS, HT and FI variables differed TP53-defined CRC subtypes using a competing risk form of Cox proportional hazards regression (25). This approach allowed us to specifically model and test the ordered interaction between a given risk factor (modeled as a covariate) and TP53 tumor status (included as a stratum variable).

Results

Informative environmental exposure and protein expression data were available for 492/563 (87%) incident CRC cases that met study criteria. Distribution by TP53 expression level included 222 (45.1%) TP53 negative, 72 (14.6%) TP53 low, and 198 (40.2%) TP53 high (Table 1). Multivariate-adjusted risk estimates for the exposures of interest and incident CRC stratified by TP53 expression are presented in Table 2. Although not statistically significant, positive associations between TP53 negative tumors and several CS-related variables were noted. For HT, a longer duration of exposure appeared to be inversely associated with risk of TP53 high tumors (RR = 0.50; 95% CI = 0.27-0.94 for > 5 years compared to ≤ 5 years exposure; p trend = 0.04) and not with TP53 negative or TP53 low tumors, although this difference in risk across expression-related subtypes did not reach statistical significance (test for heterogeneity p = 0.34). FI did not appear to influence CRC risks based on TP53 subtype.

Discussion

In this prospective cohort study of older women, we found that longer duration of HT was associated with a decreased risk for CRCs with a high TP53 protein expression level. Conversely, no statistically significant associations were observed for CS or FI and TP53-specific CRC subtypes. These data complement our previous molecular epidemiology studies of CS, HT, FI and other exposure variables with CRC subtypes defined by MSI, CIMP, *BRAF* mutation, or *KRAS* mutation status(5-6). When considered in aggregate, the IWHS molecular epidemiology data continue to support the hypothesis that CS primarily influences CRC risk through the serrated pathway (wherein TP53 mutations are uncommon) (2,26). Further investigation is needed to clarify the molecular mechanisms through which HT and FI influence colorectal carcinogenesis.

Relatively few prior studies have reported associations between the exposures of interest in the study and TP53-defined CRC subtypes. Terry et al found that heavy cigarette smoking was associated with CRC cases that did not overexpress TP53 (OR = 1.7 for current smokers and OR = 1.8 for 30 or more years of smoking) (12). While data from our study appear to be consistent with those reported by Terry et al, our relative risk estimates for CS-related variables were generally lower, and not statistically significant. Schernhammer et al reported that low FI was associated with an increased risk for colon cancers that overexpressed TP53 in a cohort study of women(11), but we were not able to replicate this result. Of note, our sample size was larger (n=492 cases; women only) and the prevalence of TP53 overexpression was higher (40%) in our study, as compared to the reports from Terry, et al. (n=157 cases; men and women; 20% with TP53 overexpression) (12) or Schernhammer, et al. (n=399 cases; women only; 36% with TP53 overexpression) (11).

Notable strengths of our study include the detailed exposure data and extended follow-up time available for IWHS subjects, central pathology review, and near-complete CRC case ascertainment. Further, CRC tissue samples were obtained for a large number of incident cases, without evidence of selection bias based on specimen availability(5,7). Relevant limitations include the restricted demographic composition of our cohort (older women), relatively small sample sizes for some of the exposure-subtype associations, and assessment of TP53 status based on IHC rather than more definitive (and resource intensive) mutation analyses. As established by others(11,24), IHC will not detect TP53 frame shift or stop codon mutations, but such abnormalities only account for about 8% of all CRC-associated mutations in the *TP53* gene.

In conclusion, our data support the possibility of heterogeneous effects of HT on TP53-related pathways of colorectal carcinogenesis in older women, although further investigation is needed given the absence of statistically significant differences across TP53-defined tumor subtypes observed in our study. Conversely, neither CS nor FI were found to be associated with CRC subtypes defined by TP53 status. Further evaluation of exposure-related CRC risks based on independent and combined molecular marker data in the IWHS cohort is ongoing, which should provide additional clarity regarding the carcinogenic mechanisms influenced by CS, HT, FI and other environmental factors.

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Panel A

Panel B

Panel C

Figure 1.
Representative examples of TP53 immunohistochemistry results (as described in Materials and Methods): negative (panel A), low (panel B), and high (panel C).

Table 1

Distributions of cigarette smoking, hormone therapy and folate intake by TP53 tumor expression among incident CRC cases.

Attribute *	TP53 Negative N=222	TP53 Low N=72	TP53 High N=198	Overall N=492
Smoking Status				
Never	139 (62.6%)	50 (69.4%)	130 (67.7%)	319 (65.6%)
Ever	83 (37.4%)	22 (30.6%)	62 (32.3%)	167 (34.4%)
Former	46 (20.7%)	15 (20.8%)	40 (20.8%)	101 (20.8%)
Current	37 (16.7%)	7 (9.7%)	22 (11.5%)	66 (13.6%)
Average Number of Cigarettes per Day				
0	139 (62.9%)	50 (69.4%)	130 (68.1%)	319 (65.9%)
1-19	42 (19.0%)	10 (13.9%)	29 (15.2%)	81 (16.7%)
20-39	35 (15.8%)	11 (15.3%)	23 (12.0%)	69 (14.3%)
40	5 (2.3%)	1 (1.4%)	9 (4.7%)	15 (3.1%)
Cumulative Pack-Years Cigarettes Smoked				
0	139 (63.2%)	50 (71.4%)	130 (68.8%)	319 (66.6%)
1-19	30 (13.6%)	9 (12.9%)	23 (12.2%)	62 (12.9%)
20-39	29 (13.2%)	8 (11.4%)	15 (7.9%)	52 (10.9%)
40	22 (10%)	3 (4.3%)	21 (11.1%)	46 (9.6%)
Hormone Therapy				
Never	143 (65.3%)	48 (67.6%)	132 (67.7%)	323 (66.6%)
Ever	76 (34.7%)	23 (32.4%)	63 (32.3%)	162 (33.4%)
Former	56 (25.6%)	17 (23.9%)	44 (22.6%)	117 (24.1%)
Current	20 (9.1%)	6 (8.5%)	19 (9.7%)	45 (9.3%)
Duration of Hormone Therapy				
Never	143 (65.9%)	48 (67.6%)	132 (67.7%)	323 (66.9%)
5 Years	55 (25.3%)	12 (16.9%)	51 (26.2%)	118 (24.4%)
> 5 Years	19 (8.8%)	11 (15.5%)	12 (6.2%)	42 (8.7%)
Folate Intake (µg/d)				
250	55 (27.0%)	13 (20.6%)	40 (22.0%)	108 (24.1%)
251-350	54 (26.5%)	17 (27.0%)	65 (35.7%)	136 (30.3%)
351-573	45 (22.1%)	22 (34.9%)	36 (19.8%)	103 (22.9%)
574	50 (24.5%)	11 (17.5%)	41 (22.5%)	102 (22.7%)

* Numbers may not sum to totals due to missing data.

Table 2

Associations of cigarette smoking, hormone therapy and folate intake with incident CRC, by TP53 tumor expression level.

Attribute	Person years	TP53 Negative		TP53 Low		TP53 High	
		N	RR (95% CI) ^a	N	RR (95% CI) ^a	N	RR (95% CI) ^a
Never Smokers	375486	139	1.00 (Ref.)	50	1.00 (Ref.)	130	1.00 (Ref.)
Ever Smokers	180409	83	1.28 (0.95,1.72)	22	1.26 (0.74,2.14)	62	1.07 (0.77,1.47)
Former	104111	46	1.16 (0.81,1.66)	15	1.39 (0.77,2.52)	40	1.16 (0.80,1.68)
Current	76297	37	1.47 (1.00,2.18)	7	1.05 (0.46,2.39)	22	0.92 (0.57,1.48)
<i>P-trend</i>			0.052		0.59		0.99
Average Number of Cigarettes per Day							
1-19	95965	42	1.20 (0.83,1.73)	10	1.00 (0.50,2.02)	29	0.92 (0.61,1.39)
20-39	73546	35	1.32 (0.88,1.96)	11	1.70 (0.86,3.36)	23	0.98 (0.61,1.56)
40	9022	5	1.69 (0.68,4.16)	1	1.48 (0.20,10.81)	9	3.49 (1.75,6.96)
<i>P-trend</i>			0.082		0.18		0.17
Cumulative Pack-Years Cigarettes Smoked							
1-19	74225	30	1.13 (0.74,1.71)	9	1.19 (0.58,2.47)	23	0.99 (0.63,1.56)
20-39	59187	29	1.38 (0.90,2.11)	8	1.42 (0.66,3.05)	15	0.74 (0.42,1.30)
40	42566	22	1.40 (0.87,2.25)	3	0.76 (0.23,2.49)	21	1.50 (0.93,2.43)
<i>P-trend</i>			0.071		0.81		0.45
Hormone Therapy							
Never	341377	143	1.00 (Ref.)	48	1.00 (Ref.)	132	1.00 (Ref.)
Ever	212696	76	0.86 (0.64,1.16)	23	0.83 (0.49,1.40)	63	0.77 (0.55,1.06)
Former	151535	56	0.84 (0.61,1.17)	17	0.86 (0.49,1.52)	44	0.70 (0.49,1.02)
Current	61161	20	0.93 (0.57,1.51)	6	0.73 (0.28,1.87)	19	0.95 (0.57,1.59)
<i>P-trend</i>			0.45		0.44		0.28
Duration of Hormone Therapy							
5 Years	148704	55	0.89 (0.64,1.23)	12	0.64 (0.34,1.22)	51	0.89 (0.63,1.25)
> 5 Years	60064	19	0.76 (0.45,1.27)	11	1.38 (0.68,2.82)	12	0.50 (0.27,0.94)
<i>P-trend</i>			0.24		0.9		0.041
Folate Intake (μg/d)							
250	142477	55	1.00 (Ref.)	13	1.00 (Ref.)	40	1.00 (Ref.)
251-350	143152	54	1.00(0.66,1.52)	17	1.49 (0.65,3.39)	65	1.58 (0.99,2.51)
351-573	142999	45	0.86(0.52,1.42)	22	2.01(0.81,4.96)	36	0.78 (0.44,1.38)
574	141705	50	1.05(0.59,1.84)	11	1.44(0.48,4.33)	41	0.98 (0.54,1.78)
<i>P-trend</i>			0.97		0.38		0.41

Relative risks (RRs) and 95% confidence intervals (CIs) based on Cox proportional hazards regression analysis. All models adjusted for body mass index (BMI), waist-to-hip ratio (WHR), physical activity level, alcohol consumption and daily intake of total calories, fat, sucrose, red meat, calcium, vitamin E, and methionine. CS analyses also adjusted for HT and FI. HT analyses also adjusted for CS, FI, age at menarche, age at menopause, OC use, and history of DM. FI analyses additionally adjusted for CS, HT and history of DM.