Short Communication

Cancer Epidemiology, Biomarkers & Prevention

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 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 colorectal cancer, can be readily assessed to differentiate biologically distinct colorectal cancer subtypes. In this prospective cohort study, we examined CS-, HT-, and FI-associated colorectal cancer 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 to 69 years, with a valid driver's license at study entry in 1986. Self-reported exposure variables were assessed at baseline. Incident colorectal cancer 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 (RR) and 95% confidence intervals (CI) for associations between CS, HT, or FI and TP53-defined colorectal cancer subtypes. Informative environmental exposure and protein expression data were available for 492 incident colorectal cancer 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 colorectal cancers (RR, 0.50; 95% CI, 0.27–0.94). No other statistically significant associations were observed. These data support possible heterogeneous effects from HT on *TP53*-related pathways of colorectal carcinogenesis in older women. *Cancer Epidemiol Biomarkers Prev; 23(2); 350–5. ©2013 AACR.*

Introduction

Colorectal cancer 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 colorectal cancer 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 colorectal cancers 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 colorectal cancer risks by TP53 expression levels (11, 12).

Somatic mutations in the *TP53* tumor-suppressor gene are reportedly found in 43% of all colorectal cancer 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

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examine associations between CS, HT, and FI with TP53-defined colorectal cancer 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 to 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 on the basis of history of any malignancy other than skin cancer ($n = 3{,}830$) or follow-up less than 1 day ($n = 10$). Additional exposure-specific exclusions were made on the basis of 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, $\geq$30 missing dietary variables, <600 calories or $\geq$5,000 calories per day, $n = 3{,}096$). 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 colorectal cancer 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). Colorectal cancer 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 colorectal cancer cases diagnosed through December 31, 2002. In total, tissue specimens were retrieved from 732 of 1,255 (58%) cases, which is similar to colorectal cancer tissue retrieval rates recently reported from the Health Professionals Follow-up Study (51%; ref. 19) and the Nurses' Health Study (58%; ref. 20). Subject demographics, exposure patterns, and tumor characteristics did not differ significantly between colorectal cancer cases with retrieved versus nonretrieved tissue specimens, as previously reported (5). All incident colorectal cancer diagnoses were confirmed by a single gastrointestinal pathologist. A total of 563 of 732 (77%) cases met the criteria for the present study (i.e., confirmed first primary colorectal cancer with sufficient tissue for the planned laboratory analyses). Paraffin blocks were serially sectioned onto 5- or 10-μm 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-μm slides were cut for H&E or IHC staining.

Characterization of TP53 protein expression by IHC

IHC for TP53 expression was performed by the Pathology Research Core at the Mayo Clinic. Briefly, slides were deparaffinized and hydrated with distilled water, antigen retrieval was done by soaking slides in EDTA in a 98°C to 100°C 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 horseradish peroxidase–labeled antibody was applied (Dako; K4061) and chromagen 3,3′-diaminobenzidine (DAB; Dako; K3468) was used and the sections were counterstained with hematoxylin. Each section or core was scored by a pathologist (T.C. Smyrk) using a combination of the staining intensity (0–3) and percentage of cells stained (0, 0%; 1, <1%; 2, 1%–10%; 3, 10%–30%; 4, 31%–67%; and 5, >67%). The two scores were added for a combined score (0–8) as reported by Harvey and colleagues (21). Each case was classified as TP53 negative if the combined score was 0, TP53 low if the score was 1 to 5 and TP53 high for a score of 6 to 8 (representative examples shown in Fig. 1). For each individual, the tumor core with the highest score was used for analysis.

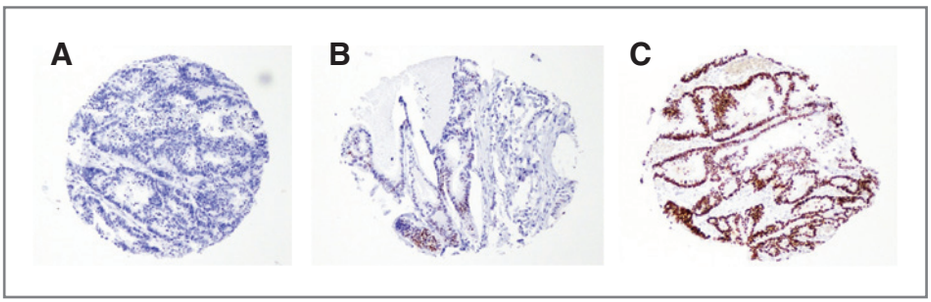

Figure 1. Representative examples of TP53 IHC results (as described in Materials and Methods): negative (A), low (B), and high (C).

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. On the basis of sequencing and IHC data for 34 cases, we found that TP53 high protein expression (Allred score $\geq$6) had 72% sensitivity and 80% specificity for detecting TP53 mutations, similar to an earlier finding reported by Curtin and colleagues (24).

Statistical analysis

Follow-up was calculated as age at completion of the baseline survey until age at first colorectal cancer 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 (RR) and 95% confidence intervals (CI) for associations between exposures of interest and colorectal cancer subtypes defined by TP53 protein expression status (negative, low, and high). For all subtype analyses, the outcome variable was incident colorectal cancer with the TP53 protein expression status of interest; all other colorectal cancer 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, oral contraceptives use, and history of diabetes mellitus. FI analyses additionally adjusted for CS, HT, and history of diabetes mellitus.

We formally determined whether risk ratios for the CS, HT, and FI variables differed in TP53-defined colorectal cancer 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 of 563 (87%) incident colorectal cancer cases that met the 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 colorectal cancer 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 seemed to be inversely associated with risk of TP53-high tumors (RR, 0.50; 95% CI, 0.27–0.94 for >5 years compared with $\leq$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 seem to influence colorectal cancer 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 of colorectal cancers with a high TP53 protein expression level. Conversely, no statistically significant associations were observed for CS or FI and TP53-specific colorectal cancer subtypes. These data complement our previous molecular epidemiology studies of CS, HT, FI and other exposure variables with colorectal

Table 1. Distributions of CI, HT, and FI by TP53 tumor expression among incident colorectal cancer cases

Attribute[a]	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%)
HT				
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 HT				
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%)
FI (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%)

[a]Numbers may not sum to totals due to missing data.

cancer 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 colorectal cancer risk through the serrated pathway (wherein *TP53* mutations are uncommon; refs. 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 colorectal cancer subtypes. Terry and colleagues found that heavy cigarette smoking was associated with colorectal cancer cases that did not overexpress TP53 (OR, 1.7 for current smokers; and OR, 1.8 for 30 or more years of smoking; ref. 12). Although data from our study seem to be consistent with those reported by Terry and colleagues, our RR estimates for CS-related variables were generally lower, and not statistically significant. Schernhammer and colleagues 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 with the reports from Terry and colleagues ($n = 157$ cases; men and women; 20% with TP53 overexpression; ref. 12) or Schernhammer and colleagues ($n = 399$ cases; women only; 36% with TP53 overexpression; ref. 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 colorectal cancer case ascertainment. Further, colorectal cancer 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

Table 2. Associations of CS, HT, and FI with incident colorectal cancer, by TP53 tumor expression level

		TP53 negative		TP53 low		TP53 high	
Attribute	**Person years**	***N***	**RR (95% CI)[a]**	***N***	**RR (95% CI)[a]**	***N***	**RR (95% CI)[a]**
Never smokers	375,486	139	1.00 (Ref.)	50	1.00 (Ref.)	130	1.00 (Ref.)
Ever smokers	180,409	83	1.28 (0.95–1.72)	22	1.26 (0.74–2.14)	62	1.07 (0.77–1.47)
Former	104,111	46	1.16 (0.81–1.66)	15	1.39 (0.77–2.52)	40	1.16 (0.80–1.68)
Current	76,297	37	1.47 (1.00–2.18)	7	1.05 (0.46–2.39)	22	0.92 (0.57–1.48)
P_{trend}			0.052		0.59		0.99
Average number of cigarettes per day							
1–19	95,965	42	1.20 (0.83–1.73)	10	1.00 (0.50–2.02)	29	0.92 (0.61–1.39)
20–39	73,546	35	1.32 (0.88–1.96)	11	1.70 (0.86–3.36)	23	0.98 (0.61–1.56)
≥40	9,022	5	1.69 (0.68–4.16)	1	1.48 (0.20–10.81)	9	3.49 (1.75–6.96)
P_{trend}			0.082		0.18		0.17
Cumulative pack-years cigarettes smoked							
1–19	74,225	30	1.13 (0.74–1.71)	9	1.19 (0.58–2.47)	23	0.99 (0.63–1.56)
20–39	59,187	29	1.38 (0.90–2.11)	8	1.42 (0.66–3.05)	15	0.74 (0.42–1.30)
≥40	42,566	22	1.40 (0.87–2.25)	3	0.76 (0.23–2.49)	21	1.50 (0.93–2.43)
P_{trend}			0.071		0.81		0.45
HT							
Never	341,377	143	1.00 (Ref.)	48	1.00 (Ref.)	132	1.00 (Ref.)
Ever	212,696	76	0.86 (0.64–1.16)	23	0.83 (0.49–1.40)	63	0.77 (0.55–1.06)
Former	151,535	56	0.84 (0.61–1.17)	17	0.86 (0.49–1.52)	44	0.70 (0.49–1.02)
Current	61,161	20	0.93 (0.57–1.51)	6	0.73 (0.28–1.87)	19	0.95 (0.57–1.59)
P_{trend}			0.45		0.44		0.28
Duration of HT							
≤5 years	148,704	55	0.89 (0.64–1.23)	12	0.64 (0.34–1.22)	51	0.89 (0.63–1.25)
>5 years	60,064	19	0.76 (0.45–1.27)	11	1.38 (0.68–2.82)	12	0.50 (0.27–0.94)
P_{trend}			0.24		0.9		0.041
FI (μg/d)							
≤250	142,477	55	1.00 (Ref.)	13	1.00 (Ref.)	40	1.00 (Ref.)
251–350	143,152	54	1.00 (0.66–1.52)	17	1.49 (0.65–3.39)	65	1.58 (0.99–2.51)
351–573	142,999	45	0.86 (0.52–1.42)	22	2.01 (0.81–4.96)	36	0.78 (0.44–1.38)
≥574	141,705	50	1.05 (0.59–1.84)	11	1.44 (0.48–4.33)	41	0.98 (0.54–1.78)
P_{trend}			0.97		0.38		0.41

NOTE: All models adjusted for BMI, 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 diabetes mellitus. FI analyses additionally adjusted for CS, HT, and history of diabetes mellitus.

[a]RRs and 95% CI based on Cox proportional hazards regression analysis.

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 colorectal cancer–associated mutations in the *TTP53* 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 colorectal cancer subtypes defined by TP53 status. Further evaluation of exposure-related colorectal cancer risks based on independent and combined molecular marker data in the IWHS cohort is ongoing, which should provide additional clarity about the carcinogenic mechanisms influenced by CS, HT, FI, and other environmental factors.

Disclosure of Potential Conflicts of Interest

N.J. Samadder has honoraria from Cook Medical, Inc. P.J. Limburg received other commercial research support from Olympus America, Fujinon, Boston Scientific, Bayer Healthcare, BENEO-Orafti, and AstraZeneca; has honoraria from Imedex; and has ownership interest (including patents) in Exact Sciences. No potential conflicts of interest were disclosed by the other authors.

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