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Associations between Environmental Exposures and Incident Colorectal Cancer by ESR2 Protein Expression Level in a Population-Based Cohort of Older Women

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Abstract

Background—Cigarette smoking (smoking), hormone therapy (MHT), and folate intake (folate) are each thought to influence colorectal cancer (CRC) risk, but the underlying molecular mechanisms remain incompletely defined. Expression of estrogen receptor beta (ESR2) has been associated with CRC stage and survival.

Methods—In this prospective cohort study, we examined smoking, MHT, and folate -associated CRC risks by ESR2 protein expression level among participants in the Iowa Women's Health Study (IWHS). Self-reported exposure variables were assessed at baseline. Archived, paraffin-embedded CRC tissue specimens were collected and evaluated for ESR2 protein expression by immunohistochemistry. Multivariate Cox regression models were fit to estimate relative risks (RRs) and 95% confidence intervals (CIs) for associations between smoking, MHT, or folate and ESR2-defined CRC subtypes.

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Results—Informative environmental exposure and protein expression data were available for 491 incident CRC cases. Positive associations between ESR2-low and -high tumors and several smoking-related variables were noted, most prominently with average number of cigarettes per day (RR = 4.24; 95% CI = 1.81–9.91 for ESR2-low and RR=2.15; 95%CI=1.05–4.41 for ESR2-high for ≥40 cigarettes compared to non-smokers). For MHT, a statistically significant association with ESR2-low tumors was observed with longer duration of exposure (RR = 0.54; 95% CI = 0.26–1.13 for > 5 years compared to never use). No associations were found for folate.

Conclusions—In this study, smoking and MHT were associated with ESR2 expression patterns.

Impact—These data support possible heterogeneous effects from smoking and MHT on ERβ-related pathways of colorectal carcinogenesis in older women.

INTRODUCTION

Colorectal cancer (CRC) represents the third most common incident and fatal cancer in the United States (with estimates of 136,830 new cases and 50,310 attributable deaths in 2014) (1). Cigarette smoking has been shown by us and others to increase the risk for CRC (2–4), while hormone therapy (MHT) has protective effects (5–8). Less clear is the role that folate intake has on CRC risk (9). Kim et al found an increase in folate modestly decreased risk, although other studies have yielded mixed results (10–11).

Molecular heterogeneity in colorectal carcinogenesis is well established (12–14). Concordantly, emerging data from our group and others demonstrate differential associations between common environmental exposures, including smoking, MHT and folate, and incident CRCs defined by microsatellite instability (MSI), CpG island methylator phenotype (CIMP), *KRAS* and *BRAF* mutation status (2–3,15–18) and TP53 protein expression (19), among other phenotypic markers. Most significantly, post-menopausal MHT was associated with a lower risk for MSI-L/MSS tumors (15) and smoking was shown to be associated with MSI-high, CIMP-positive, and BRAF-mutated tumors (2).

To date, relatively few studies have examined subtype-specific CRC risks by ESR2 (ERβ) expression levels (20–21). ESR2 (ERβ) is the main estrogen receptor expressed in colon tissue (22). Although the exact mechanism is yet to be determined, it appears ESR2 signaling has a role in the protective effect of MHT against colon tumor development (23). ESR2 is highly expressed in normal colonic mucosa but declines in colon adenocarcinoma. ESR2 loss in colon tissue is associated with progressing cancer and cell dedifferentiation (24–25) as well as advanced cancer stage and poor survival (26). Both tobacco carcinogens and estrogen utilize some of the same enzymes for metabolites. Smoking induces the expression of genes that are involved in estrogen metabolism and, in lung tissue, has been shown to increase the carcinogenic estrogen metabolite 4-OHE. So it seems biologically plausible that their pathways may overlap and smoking may influence the estrogen pathway (27). Further clarification of the risk factors for molecularly defined CRC subtypes could inform more targeted prevention, early detection, and treatment strategies.

In this current study we used baseline data and archived tumor tissue specimens from the prospective Iowa Women's Health Study (IWHS) to examine exposures associated with

ESR2-defined CRC subtypes in older women. smoking, MHT, and folate were investigated as potentially modifiable lifestyle, medication, and dietary factors, respectively. Based on previous reports from our group and others (2–3, 15–16, 18–19), these exposures may be plausibly linked to heterogeneous pathways of colorectal carcinogenesis.

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 (28). Briefly, a 16-page baseline questionnaire was used to collect comprehensive self-reported demographic, dietary, lifestyle, and medication data from 41,836 Iowa women, ages 55–69 years, who held a valid driver's license at baseline in 1986. Subjects were excluded for the present study based on the following factors (not mutually exclusive): history of any malignancy other than skin cancer (n=3830); follow-up less than one day (n=10); incomplete baseline exposure information (n=660 for smoking and n=200 for MHT); incomplete premenopausal or menopause status (for MHT analyses only, n=569); or invalid dietary data (for folate analyses only, ≥ 30 missing dietary variables, self-reported intakes of < 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

Smoking 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 (29). Folate 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 MHT and duration of MHT exposure were also collected, as described previously (15). 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 (30). 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 (31–32).

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%) (33) and the Nurses' Health Study (58%) (34) Women with tissue available for ER analysis were slightly older than those for whom tissue was not available (mean 73.9 vs. 72.1 years of age). Otherwise, subject demographics, exposure patterns, and tumor characteristics did not differ significantly between CRC cases with retrieved versus non-retrieved tissue specimens. 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 to 5 or 10 um slices and placed on 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 and two normal cores were taken from each block and placed into a tissue microarray (TMA) block along with liver controls. The TMA was produced by the Mayo Clinic Pathology Research Core lab using the Beecher ATA-27 automated array. From the TMA 5 um sections were cut and placed on slides for H&E or IHC staining.

Characterization of ESR2 protein expression by immunohistochemistry

IHC for ESR2 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 a 98–100° steamer for 30 minutes. A protein block was applied (DAKO X0909) and the primary antibody (Estrogen Receptor beta antibody, clone PPG5/10 (Thermo Scientific # MA1-27412) at 1:25 dilution) was applied. The secondary HRP--labeled antibody was applied (DAKO K4061), chromagen DAB (DAKO K3468) was used, and the sections were counterstained with hematoxylin. Breast cancer tissue was used as a positive control and liver tissue for negative controls. 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 to10%, 3–10 to 30%, 4-31-67%, 5–>67%). The two scores were added for a combined score (0–8) as reported by Harvey et al. Each case was classified as ESR2-negative if the combined score was 0, ESR2-low if the score was 1–5 and ESR2-high for a score of 6–8 (35) (representative examples shown in Figure 1). For each individual, the tumor core with the highest score was used for analysis.

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 ESR2 protein expression status (negative, low, and high). Incidence was modeled as a function of age rather than time on study, since age is a better predictor of CRC risk on our cohort than follow-up time. (36) Smoking was examined by overall status (never, ever, former, or current), average number of cigarettes smoked per day, and cumulative cigarette pack-years; MHT was examined by overall status (never, ever, former, or current) and duration of use; and folate was 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 (for example, never, former and current smoking groups for smoking status) and including the resulting variable as a linear term in the Cox regression model. Multivariable adjustments were applied. All models were 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. Smoking analyses were also adjusted for MHT and folate. MHT analyses were also adjusted for smoking, folate, age at menarche, age at menopause, OC use, and history of DM. Folate analyses were additionally adjusted for smoking, MHT and history of DM.

For all subtype analyses, the outcome variable was incident CRC with the ESR2 protein expression status of interest; all other CRC cases (including those with missing or unknown ESR2 status) were considered censored observations at the date of diagnosis. We determined if risk ratios for smoking, MHT, and folate differed according to these cancer subtypes using a competing risk form of Cox proportional hazards analysis (37). This approach allowed us to model and test the interaction between an exposure (modeled as a covariate) and molecular/tumor subtype (modeled as a stratum variable).

Results

Informative environmental exposure and protein expression data were available for 491/563 (87%) incident CRC cases that met study criteria. Distribution by ESR2 expression level included 66 (13%) ESR2-negative, 126 (26%) ESR2-low, and 299 (61%) ESR2-high (Table 1). Multivariate-adjusted risk estimates for the exposures of interest and incident CRC stratified by ESR2 expression are presented in Table 2. We found positive associations between ESR2-low and -high tumors and several smoking-related variables when looking at those that measured the quantity of smoking (cigarettes per day and pack-years). The most significant of these was with the average number of cigarettes per day. Both ESR2-low and -high had p-trends of 0.02 and elevated RRs for >40 cigarettes per day compared to never smokers (RR = 4.24; 95% CI = 1.81–9.91 for ESR2-low and RR = 2.15; 95% CI = 1.05–4.41 for ESR2-high). For cumulative pack-years of cigarettes smoked, a statistically significant association was seen for ≥ 40 pack-years in ESR2-low tumors (RR = 1.88; 95% CI = 1.05–3.36 compared to never smokers; p-trend = 0.04), and a marginally significant association was seen for ESR2-high tumors (RR = 1.42; 95% CI = 0.94–2.14; p-trend = 0.06). No associations with smoking were observed for ESR2-negative tumors. Although point estimates for the associations with smoking were larger for ESR2-low and -high tumors than for ESR2-negative CRC, tests for heterogeneity in these associations failed to reach statistical significance (p >0.20 for each), acknowledging low power for this test

For MHT, a statistically significant, inverse association with ESR2-low tumors was observed when comparing never use to former (RR=0.68; 95% CI 0.44–1.07) and current (RR = 0.59; 95% CI = 0.28–1.23) use of MHT (p-trend=0.05); there was also a trend with longer duration of MHT exposure (RR = 0.54; 95% CI = 0.26–1.13 for > 5 years compared to no exposure; p-trend = 0.04). Similar trends were observed in the ESR2-negative tumors, but numbers were very small (4 cases with current or >5 years use). No associations with MHT were observed for ESR2-high tumors. As with the smoking analyses, tests for heterogeneity in subtype-specific MHT associations did not reach statistical significance (p > 0.40).

Folate intake was not associated with CRC risks, either overall or for any ESR2 subtype.

Discussion

In this prospective cohort study of older women, we found that increased smoking exposure appeared to influence ESR2-low and ESR2-high CRCs to a greater degree than ESR2-negative tumors (although sample size was limited in some smoking categories and tests for heterogeneity were not statistically significant). Additionally, longer duration of MHT use was associated with a decreased risk for CRCs with ESR2-low and, to a lesser extent, ESR2-negative protein expression levels. Conversely, no statistically significant associations were observed for folate and ESR2-specific CRC subtypes. These novel data add to the body of literature from our previous molecular epidemiology studies of smoking, MHT, folate and other exposure variables with CRC subtypes defined by MSI, CIMP, *BRAF* mutation,p53 protein expression or *KRAS* mutation status (2–3, 15–16, 18–19).

Coupled with our previously published results, the IWHS molecular epidemiology data reported herein continue to support the hypothesis that smoking primarily influences CRC risk through the serrated pathway (12, 38–40). The serrated pathway appears to be initiated by BRAF mutation and progresses through a serrated precursor (sessile serrated adenoma) to cancers characterized by mutant BRAF, high CIMP and, often, high MSI. Burnett-Hartman et al found that serrated polyps were positively associated with cigarette smoking (41). Our group previously reported that smoking was associated with CIMP positive, *BRAF* mutated and MSI high tumors, linking this lifestyle habit to the serrated pathway of colorectal carcinogenesis (2). Interestingly, in lung tissue, smoking induces expression of CYP1B1, an enzyme that metabolizes both the tobacco carcinogens and estrogen and smoking also increases the carcinogenic estrogen metabolites (4-OHE). Some of the estrogen metabolites that are produced are known to activate the ER-mediated signaling pathways (27). Cleary et al found a significant interaction between smoking status and CYP1B1 and other carcinogen metabolism gene variants in CRC (42). Together, these findings provide a biologically credible mechanism for the smoking-related risk associations observed in the IWHS cohort.

MHT has been shown to provide a protective effect on CRC risk (5–8, 15, 23). In our previous work we found MHT may reduce CRC risk in *KRAS-WT* tumors in the distal colorectum (16). We also found MHT to be associated with a decreased risk for MSI-L/MSS tumors, and longer duration MHT use decreased the risk for CIMP-negative and BRAF-WT tumors (15). These results seem to indicate that MHT influences CRC risk through the

traditional pathway as defined by Leggett and Whitehall (12). In this current study, we found longer duration use of MHT was associated a decreased CRC risk in ESR2-low expressing tumors. ESR2 has action against cancer growth and is increased with E2 through the p38/MAPK pathway in DLD-1 colon cancer cells (25). Since the loss of ESR2 is associated with more advanced stages of CRC this could be a mechanism for the protective effect of MHT on ESR2-low expressing tumors. Further work is required to determine the exact effect of increased ESR2 and its transcriptional mechanism, and how MHT exposure may influence colorectal carcinogenesis through this pathway.

Our group previously reported no significant associations between folate intake and incidence CRC after adjustment for potential confounding factors, either overall or within molecular subtypes of MSI, CIMP, BRAF, p53, or KRAS status (18–19). In the current study we also found no association between folate and CRC risk based on ESR2 status. Further work is needed to determine the molecular mechanism for the possible protective effects of folate intake on CRC risk.

Relatively few prior studies have reported associations between the exposures of interest in this study and ESR2-defined CRC subtypes. Rudolph et al found that CRC risk was significantly reduced with ESR2-positive tumor with current and longer duration MHT. Like our study, heterogeneity of association according to ESR2 status was not statistically significant (20). While we saw reduced CRC risk only with ESR2-low samples, it is hard to compare the results because we had a more complex category scale. It appears that our ESR2-low cases would fall into Rudolph's ESR2-negative group (less than 10% strong staining or less than 50% weak staining). In both studies there was the same correlation with at least some ESR2 expression. Although our population groups appear to be similar, there may be some subtle differences due to location, culture or treatment protocol (Germany vs Iowa). If we combine our ESR2-low and -negative categories, we have a higher proportion of patients with ESR2-high expression than Rudolph et al (61% vs 51%). We also used a different antibody in our study. Rudolph et al used the 14C8 clone which targets the N terminus of the protein while our study used the PPG5/10 clone which targets the C terminus of the protein. According to Skliris et al, 14C8 and PPG5/10 showed nearly equivalent results in the staining of FFPE tissue (43). However, the importance of other ESR2 isoforms (5 reported) is currently unknown (44) so their differential staining by either antibody may affect the results as it is not yet established whether MHT interacts with all of them in the same way. Future experiments to determine how estrogen interacts with the different variants could be useful in determining the mechanism for their protective effect.

We evaluated nuclear staining, but there are indications that cytoplasmic staining may also be informative. Several groups noticed a difference between normal tissue and tumor tissue with the ESR2 staining location. Normal tissue tended to have all nuclear staining while tumor tissue had both nuclear and cytoplasmic staining (22, 44). Examining this could help explain the mechanism for loss of ESR2 protein in some tumors. Traditionally, estrogen receptors are located in the nucleus where they bind to estrogen and modulate gene expression. There are also reports of plasma membrane estrogen receptors that induce more rapid signaling (45–46).

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. Use of the molecular pathological epidemiology study design (47) permitted more focused evaluation of CRC subtype-specific exposure associations, with accompanying mechanistic inferences. As cautioned by Ogino et al., selection bias can be introduced into molecular pathological epidemiology studies if the analyzed tumor samples are not representative of the broader subject cohort or target population from which they were derived (47). In our study, we retrieved tissue samples from 58% of the CRC cases requested (similar to other large cohort studies), without evidence of selection bias based on specimen availability (2, 15). By using TMAs for our IHC analyses, we were also able to stain many more samples with normal and tumor cores, along with replicates that wouldn't have been feasible to assess using a whole section approach, and reduce the run to run variability that would have been present had each case been immunostained separately.

The restricted demographic composition of our cohort (older midwest women) and the relatively small sample sizes for some of the exposure-subtype associations are relevant limitations to our study. This can be seen in our tests for heterogeneity and with some of the association trends that didn't reach statistical significance, likely due to lack of sufficient power. This is also evidenced by the large confidence intervals in some of our comparisons performed with limited sample numbers in category.

Additionally, although we utilized a very extensive questionnaire, our study was still dependent on patient recall for the analyzed exposure information, which may not be as reliable as the molecular assay data. As discussed, the assessment of ESR2 status based on IHC results with one antibody rather than a more comprehensive (and resource intensive) antibody panel to look at different isoforms should be considered when interpreting our results (42).

In conclusion, our data support the possibility of heterogeneous effects of MHT and smoking on ESR2-related pathways of colorectal carcinogenesis in older women, while no clear association between folate exposures and ESR2-defined CRC subtypes was observed. These findings continue to support the hypothesis that smoking primarily influences CRC risk through the serrated pathway. 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 smoking, MHT, folate and other environmental factors.

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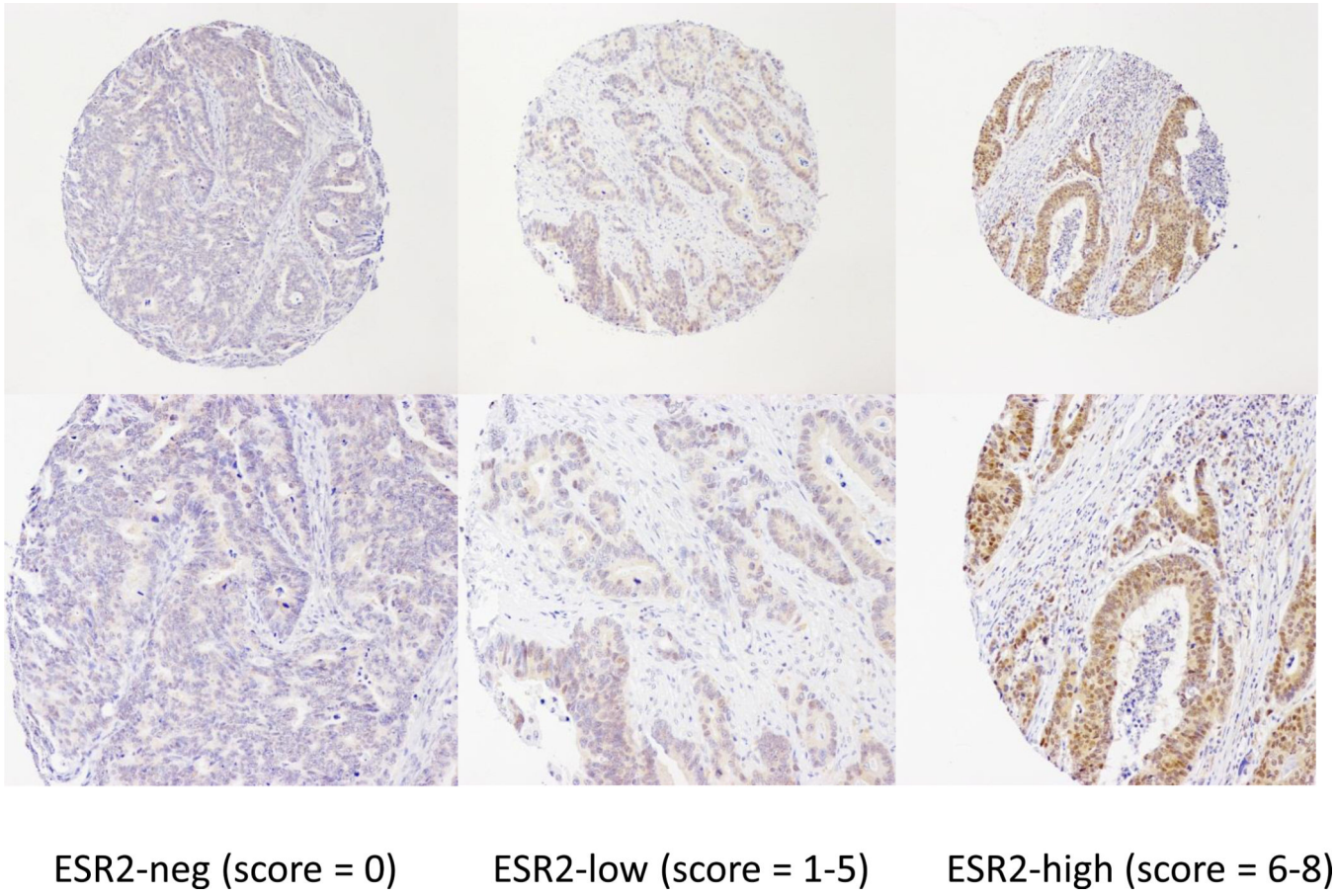

Figure 1.
Classification of ESR2 protein expression in colorectal cancer TMA cores

Table 1

Distributions of cigarette smoking, hormone therapy and folate intake by ESR2 tumor expression among incident CRC cases.

Attribute*	ESR2 Negative N=66	ESR2 Low N=126	ESR2 High N=299	Overall N=491
Age at Baseline: mean (SD)	63.8 (4.3)	63.0 (3.8)	62.9 (4.1)	63.1 (4.1)
Age at CRC Diagnosis: mean (SD)	73.6 (6.3)	73.9 (5.9)	73.9 (5.9)	73.9 (5.9)
Smoking Status				
Never	47 (71.2%)	77 (61.6%)	191 (65%)	315 (64.9%)
Ever	19 (28.8%)	48 (38.4%)	103 (35%)	170 (35.1%)
Former	14 (21.2%)	29 (23.2%)	59 (20.1%)	102 (21%)
Current	5 (7.6%)	19 (15.2%)	44 (15%)	68 (14%)
Average Number of Cigarettes per Day				
0	47 (72.3%)	77 (61.6%)	191 (65.2%)	315 (65.2%)
1–19	10 (15.4%)	23 (18.4%)	49 (16.7%)	82 (17%)
20–39	7 (10.8%)	19 (15.2%)	45 (15.4%)	71 (14.7%)
≥ 40	1 (1.5%)	6 (4.8%)	8 (2.7%)	15 (3.1%)
Cumulative Pack-Years Cigarettes Smoked				
0	47 (72.3%)	77 (62.6%)	191 (65.9%)	315 (65.9%)
1–19	10 (15.4%)	16 (13%)	36 (12.4%)	62 (13%)
20–39	3 (4.6%)	15 (12.2%)	35 (12.1%)	53 (11.1%)
≥ 40	5 (7.7%)	15 (12.2%)	28 (9.7%)	48 (10%)
Hormone Therapy				
Never	41 (62.1%)	87 (70.2%)	196 (66.7%)	324 (66.9%)
Ever	25 (37.9%)	37 (29.8%)	98 (33.3%)	160 (33.1%)
Former	21 (31.8%)	27 (21.8%)	67 (22.8%)	115 (23.8%)
Current	4 (6.1%)	10 (8.1%)	31 (10.5%)	45 (9.3%)
Duration of Hormone Therapy				
Never	41 (62.1%)	87 (70.7%)	196 (67.1%)	324 (67.4%)
≤ 5 Years	21 (31.8%)	26 (21.1%)	68 (23.3%)	115 (23.9%)
> 5 Years	4 (6.1%)	10 (8.1%)	28 (9.6%)	42 (8.7%)
Folate Intake (μg/d)				
≤ 250	16 (25.8%)	23 (20%)	70 (25.8%)	109 (24.3%)
251–350	17 (27.4%)	35 (30.4%)	82 (30.3%)	134 (29.9%)
351–573	14 (22.6%)	21 (18.3%)	66 (24.4%)	101 (22.5%)
≥ 574	15 (24.2%)	36 (31.3%)	53 (19.6%)	104 (23.2%)

*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 ESR2 tumor expression level.

Attribute	Person years	ESR2 Negative		ESR2 Low		ESR2 High	
		N	RR (95% CI)[a]	N	RR (95% CI)[a]	N	RR (95% CI)[a]
Never Smokers	375486	47	1.00 (Ref))	77	1.00 (Ref)	191	1.00 (Ref)
Ever Smokers	180409	19	0.93 (0.53,1.64)	48	1.35 (0.91,2.00)	103	1.24 (0.96,1.61)
Former	104111	14	1.23 (0.67,2.28)	29	1.28 (0.81,2.03)	59	1.19 (0.87,1.61)
Current	76297	5	0.54 (0.21,1.40)	19	1.46 (0.86,2.50)	44	1.33 (0.94,1.90)
P-trend			*0.40*		*0.12*		*0.08*
Average Number of Cigarettes per Day							
1–19	95965	10	0.91(0.45,1.85)	23	1.14 (0.69,1.87)	49	1.10 (0.79,1.52)
20–39	73546	7	0.82 (0.36,1.86)	19	1.38 (0.81,2.37)	45	1.35 (0.95,1.91)
≥40	9022	1	1.01 (0.14,7.38)	6	4.24 (1.81,9.91)	8	2.15 (1.05,4.41)
P-trend			*0.66*		*0.02*		*0.02*
Cumulative Pack-Years Cigarettes Smoked							
1–19	74225	10	1.27 (0.63,2.55)	16	0.99 (0.55,1.78)	36	1.09 (0.76,1.58)
20–39	59187	3	0.44 (0.13,1.43)	15	1.30 (0.72,2.34)	35	1.26 (0.86,1.84)
≥ 40	42566	5	0.90 (0.35,2.33)	15	1.88 (1.05,3.36)	28	1.42 (0.94,2.14)
P-trend			*0.46*		*0.04*		*0.06*
Hormone Therapy							
Never	341377	41	1.00 (Ref)	87	1.00 (Ref)	196	1.00 (Ref)
Ever	212696	25	0.92 (0.54,1.56)	37	0.66 (0.44,0.99)	98	0.82 (0.63,1.07)
Former	151535	21	1.02 (0.58,1.77)	27	0.68 (0.44,1.07)	67	0.73 (0.54,0.99)
Current	61161	4	0.63 (0.22,1.78)	10	0.59 (0.28,1.23)	31	1.11 (0.74,1.64)
P-trend			*0.53*		*0.05*		*0.52*
Duration of Hormone Therapy							
≤ 5 Years	148704	21	1.14 (0.66,1.97)	26	0.69 (0.44,1.09)	68	0.80 (0.60,1.08)
> 5 Years	60064	4	0.41 (0.12,1.35)	10	0.54 (0.26,1.13)	28	0.89 (0.59,1.34)
P-trend			*0.36*		*0.04*		*0.25*
Folate Intake (μg/d)							

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Attribute	Person years	ESR2 Negative		ESR2 Low		ESR2 High	
		N	RR (95% CI)[a]	N	RR (95% CI)[a]	N	RR (95% CI)[a]
≤ 250	142477	16	1.00 (Ref)	23	1.00 (Ref)	70	1.00 (Ref)
251–350	143152	17	1.30 (0.61,2.77)	35	1.65 (0.91,3.00)	82	1.09 (0.75,1.58)
351–573	142999	14	1.37 (0.56,3.32)	21	1.24 (0.60,2.56)	66	0.76 (0.49,1.18)
≥ 574	141705	15	1.48 (0.52,4.16)	36	2.09 (0.97,4.54)	53	0.73 (0.44,1.20)
P-trend			*0.46*		*0.12*		*0.11*

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. Smoking analyses also adjusted for MHT and folate. MHT analyses also adjusted for smoking, FI, age at menarche, age at menopause, OC use, and history of DM. folate analyses additionally adjusted for smoking, MHT and history of DM.