Hormone therapy, DNA methylation and colon cancer

Anna H. Wu*, Kimberly D. Siegmund, Tiffany I. Long¹, Wendy Cozen, Peggy Wan, Chiu-Chen Tseng, Darryl Shibata¹,² and Peter W. Laird¹,³

Department of Preventive Medicine, ¹Department of Surgery, ²Department of Pathology and ³Department of Biochemistry and Molecular Biology, Keck School of Medicine, University of Southern California, 1441 Eastlake Avenue, Los Angeles, CA, USA

*To whom correspondence should be addressed. Tel: +1 323 865 0484; Fax: +1 323 865 0139; Email: annawu@usc.edu

Observational epidemiological studies and randomized trials have reported a protective effect of estrogen and progestin therapy (EPT) on the risk of colorectal cancer but the findings on estrogen-alone therapy (ET) are less consistent. The mechanism by which menopausal hormones influence risk of colorectal cancer has not been well studied. To further investigate the relationship between menopausal hormones and risk of colon cancer, we conducted a population-based case-control study in Los Angeles County involving 831 women with newly diagnosed colon cancer and 755 population-based control women. Risk of colon cancer decreased significantly with increasing duration of current use of EPT (2) but risk was unrelated to use of ET or EPT. We explored if current use of menopausal hormones is associated with DNA methylation of estrogen receptor (ESRI and ESRI), progesterone receptor and other genes in the colonic tissues of a subset of colon cancer patients (n = 280) we interviewed. Our results suggest that current menopausal hormone users compared with non-current users displayed increased DNA methylation of progesterone receptor in the ‘normal’ colonic tissues (P = 0.055) and increased DNA methylation of ESRI in the ‘tumorous’ colonic tissues (P = 0.056). These findings on DNA methylation and hormone therapy use need confirmation in larger studies.

Introduction

Since the early 1980s, evidence has accumulated to suggest that use of exogenous hormones may reduce the risk of colorectal cancer. In 1999, a meta-analysis of 18 observational epidemiological studies concluded that the risk of colorectal cancer is reduced, particularly in association with current use of hormone therapy (HT) (1). Most of the observational studies included in this meta-analysis were conducted when estrogen-alone therapy (ET) was commonly used; only 3 of the 18 studies had information on use of estrogen and progesterin therapy (EPT) and thus, the inverse association was found largely in relation to use of ET (1). In the Women’s Health Initiative (WHI) clinical trial study, risk of colorectal cancer was not reduced in women randomized to EPT compared with those randomized to placebo (4). This protective effect of exogenous menopausal hormones raises the possibility that higher levels of endogenous estrogen may be associated with a reduced risk of colorectal cancer. Contrary to hypothesis, higher levels of endogenous estrone (5) and estradiol (6) were associated with elevated risk of colorectal cancer in two recent case-control studies nested within prospective studies.

We conducted a population-based case-control study of colon cancer in Los Angeles County in the late 1990s. Our primary objective was to further investigate the role of ET versus EPT on risk of colon cancer. The mechanisms by which use of HT may protect against the development of colorectal cancer are not known. Thus, a secondary objective of our study was to determine if use of HT is associated with DNA methylation of the estrogen receptor (ESR) gene and related genes. Based on the results reported by Issa et al. (7) on ESR DNA methylation, we hypothesized that DNA methylation levels of ESR (and related genes) in colonic tissues of patients with colon cancer may be lower in HT users compared with non-HT users. We report our results herein.

Materials and methods

Study population

This was a population-based case-control study of colon cancer in Los Angeles County. The cases were identified by the Los Angeles County Cancer Surveillance Program, part of the National Cancer Institute’s Surveillance, Epidemiology and End Results Program, covering all residents of Los Angeles County. Eligible patients were English-speaking women with a histologically confirmed primary colon cancer diagnosed between the ages of 55 and 74 years on or after January 1998 through December 2002 and who were residents of Los Angeles County at the time of diagnosis. A total of 1868 eligible case patients were identified; 438 cases died prior to interview and we were unable to contact another 239 cases because they had moved away from Los Angeles County and could not be located even after repeated efforts, including home visits. Of the remaining 1191 cases, 332 patients refused to participate, resulting in 859 complete interviews. The response range varied between 72% (for cases approached) and 46% among all cases identified. We extracted stage of colon cancer at diagnosis from Cancer Surveillance Program registry records.

Controls were identified through a well-established neighborhood recruitment algorithm, which we have used successfully in previous studies of breast, endometrial, ovarian and other cancers to investigate the role of hormonal and non-hormonal medications and other factors (8). This algorithm defined a specified sequence of houses to be visited in the neighborhoods where index cases lived at the time of diagnosis. We sought to interview as the control the first eligible resident in the sequence. If the first eligible control subject refused to participate, the second eligible one in the sequence was asked and so on. Letters were left when no one was home, and follow-up was by mail and telephone; further visits to the neighborhood continued until either an eligible control agreed to be interviewed or 150 housing units had been screened. When we failed to identify an exact race/ethnicity-matched control, we accepted a control who was matched on age. A total of 755 control women were successfully interviewed by the closing date of the study. The first eligible match was interviewed for 69% of the patients, the second match for another 19% and the third or later match for 11% of the patients.

Data collection

In-person interviews were conducted using a standardized structured questionnaire. To achieve our main study objective, which was to evaluate the role of exogenous hormones, detailed question on use of HT was asked. Specifically, for each episode of hormone use; brand names; dosage and temporal factors, including age of first use, age of last use, total duration of use, side effects and reasons for stopping, were asked. During the interview, a book of color photographs of exogenous hormones marketed in the USA was used as a visual aid to identify hormone used by a respondent. In addition, participants were interviewed using a comprehensive questionnaire that covered medical, menstrual and reproductive history, use of select non-hormonal medications; body size; physical activity and family history of colorectal and other cancers. A reference date was defined as 1 year before the date of diagnosis of the case. This same reference date was used for each case’s matched control subject. Calendars were used to chart major life events and reproductive and contraceptive histories.

Abbreviations: 95% CI, 95% confidence interval; BMI, body mass index; EPT, estrogen and progestin therapy; ESRI, estrogen receptor; ET, estrogen-alone therapy; HT, hormone therapy; OC, oral contraceptive; OR, odds ratio; PMR, percentage of fully methylated reference; RR, relative risk; WHI, Women’s Health Initiative.

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DNA methylation
Microdissection and DNA extraction. At the interview, subjects were asked to sign a medical release, which allowed access to their diagnostic tumor block materials. Approximately 95% of patients interviewed consented to the medical release. Among those who consented, we used the Slide Retrieval Component of the Tissue Procurement Core Resource of the University of Southern California/Norris Comprehensive Cancer Center to obtain relevant tumor blocks and/or unstained and formalin-fixed slides. When we receive the tumor blocks, 5 μ sections lightly stained with hematoxylin and eosin for each sample were prepared and examined by our study pathologist (D.S.) to verify and localize the colorectal adenocarcinoma tissue. The adenocarcinoma tissues were then carefully microdissected from other cell types from an adjacent section.

Sodium bisulfite conversion. The DNA from the paraffin tissues was lysed in a solution containing 100 nM Tris, 10 nM EDTA, proteinase K (milligrams per milliliter) and transfer RNA (0.05 mg/ml) overnight at 50°C. Sodium bisulfite conversion of genomic DNA was performed as described previously (9,10). The DNA was incubated for 16 h at 50°C in dark to ensure complete conversion. After sodium bisulfite conversion, the methylation analysis was performed by the fluorescence-based real-time polymerase chain reaction assay, MethyLight, as described in detail (11–13). Briefly, three sets of primers and probes designed specifically for bisulfite-converted DNA were used to detect the bisulfite modification of the specific gene of interest and gene controls for β-actin (ACTB) and collagen 2A1 (COL2A1) which were used to normalize for the amount of input DNA for each reaction. SsI-treated human peripheral blood leukocyte DNA was used as a fully methylated reference and SsI-treated leukocyte DNA as a control sample (SsI-treated leukocyte DNA) and multiplying by 100. This was repeated for COL2A1, the second control gene, and the two values were averaged to obtain the PMR (14).

DNA methylation analysis. A panel of seven genes (ESR1, ESR2, PGR, MLH1, CDKN2A, MGMT and MYOD1) were included in our DNA methylation sub-study using MethyLight (see above). In addition to studying ESR1, which was implicated in the study by Issa et al. (7), we also studied ESR2, which was discovered in 1998 (15,16), and PGR because it has a critical part in the estrogen signaling pathway (17) and has been shown to be methylated in colon and hormone-related tumors, such as breast cancer (18). The other genes were selected because they are known to be hypermethylated in colorectal adenocarcinomas and/or adenomas and because of its known relevance to the development of colorectal cancer (19).

The study was approved by the Institutional Review Board of the Keck School of Medicine at the University of Southern California. Informed consent was obtained from each case and control before her interview.

Statistical analysis
By study design, lifestyle information of controls was counted up to the comparable calendar time of the matching case’s reference date (i.e. diagnosis date). This analysis was restricted to women who were postmenopausal, defined as menstrual period has stopped for at least 6 months before her diagnosis date (for cases) or reference date (for controls). For naturally menopausal women, age at menopause was estimated as follows: natural menstruation was taken to mean menstruating and not using oral contraceptives (OCs) or menopausal HT at the time. For women who started HT before their last menstrual period, we set age at menopause as the age at which they started HT use because we assumed that HT was taken for menopausal symptoms; their menopausal status is classified as ‘HT menopause’. For women taking OCs, age at menopause was taken at the end of the period of OC use if no ‘natural’ menstruation occurred thereafter. For women who had a bilateral oophorectomy, their age at ‘surgical menopause’ is the age at oophorectomy. For women (172 cases and 151 controls) who had undergone a ‘simply hysterectomy’ (i.e. without a bilateral oophorectomy), their ages at menopause are not truly known.

We examined HT use around the time of menopause separately for ET and EPT. Recency of HT use (current or former) and duration of ET and EPT use were calculated for each postmenopausal subject. A subject who used both ET and EPT contributed duration of years use of both types of regimen as these variables were mutually adjusted for each other in the analysis.

Of the 856 cases and 779 controls we interviewed, final results included 831 cases and 755 controls. We excluded 25 case patients and 24 control subjects from the final analyses because they were premenopausal and had previous cancer or missing information on body size, menstrual or pregnancy history or one of the other adjustment covariates. We utilize all the cases and controls interviewed and calculated odds ratios [ORs; relative risk (RR) estimates], their corresponding 95% confidence intervals (95% CIs) and P values by unconditional logistic regression methods with adjustment for reference age (≤61, 61–65, 66–70 and >70+ years), race/ethnicity (non-Hispanic White, African-American, Hispanic-American and Asian-American), education (less than high school, high school, some college or college graduate), menopausal status (natural menopause, HT menopause, bilateral oophorectomy and simple hysterectomy) and age at menopause (<40, 40–44, 45–49, 50–54 and 55+ years; Model A). A more elaborate regression model included other covariates, including body mass index (BMI) (kg/m²) (<25.2, ≥25.2–26.2, ≥26.2–30.3 and ≥30.3), parity (0, 1, 2, 3, 4 and 5+), use of OCs (years), duration of regular alcohol intake (never, 1–20, 21–40 and >40+ years), duration of regular smoking (never, 1–20, 21–40 and >40+ years), family history of colorectal cancer (no or yes), use of aspirin (years), physical activity (years) and screening for colorectal cancer (Model B). For HT, BMI and other relevant exposures, tests for trend (P values) were performed by coding each variable as a grouped linear variable (i.e. 1, 2, 3, etc.) To examine the potential effect modification of the ET (or EPT)–cancer association by BMI or other covariates, interaction terms for specific HT–body size and other measures were tested.

In this cross-sectional analysis on DNA methylation profile, each subject was assigned two PMR scores for the level of methylation at each of the seven CpG islands, one for the adenocarcinoma tissue (tumorous) and one for the adjacent normal colonic mucosal tissue (normal). We obtained tissue microarrays from 280 patients: all had ‘tumorous’ colonic tissues and 234 patients had ‘normal’ colonic tissues for DNA methylation analysis. A region was considered ‘tumorous’ if at least 70% of the cells were malignant, whereas a region was considered ‘normal’ if 100% of the cells were considered non-malignant. We used the unconditional logistic regression method to examine the association between DNA methylation and HT use with adjustment for age, race/ethnicity, batch and colon subtype. On the basis that risk of colon cancer was reduced in association with current HT use, we treated cases who were current HT users as ‘exposed’ and non-current users as ‘unexposed’ in our DNA methylation analysis. Our expectation was that any effect of HT on DNA methylation would be reflected in a higher (or lower) level of methylation in current HT users compared with non-current HT users. Adjusted ORs and corresponding 95% CIs were reported (Tables IV and V). The PMR is a relative measure and we converted the PMR for each gene as no/low, medium and high methylated based on the distribution of PMR values. We used cutoffs that were close to tertiles when possible but for some genes (e.g. ESR1 and MYOD1), the highest levels represented the upper two quartiles, whereas the lower two quartiles represented the no/low and medium level, respectively. For sensitivity analysis, we repeated analyses using uniform cutoffs of 0, >0–5, >5–50 and >50 PMR for all genes under study but combined the data from two categories if there were no or too few individuals in a specific level. In addition, we constructed a score by summing three individual scores assigned to ‘high/medium’ versus ‘low’ methylation levels of ESR1, ESR2 and PGR, the three genes directly responsive to HT; Low methylation levels were scored as 0, whereas medium/high methylation levels were assigned a score of 1. Using the same method, we constructed a total score to summarize methylation levels across all seven genes we investigated. Separate analyses were conducted for DNA methylation in ‘normal’ and ‘tumorous’ colonic tissues (Tables IV and V, respectively). P values ≤5% are considered statistically significant and all P values quoted are two sided. Statistical analyses were carried out using established statistical packages, including SAS (9.13) and EPILOG Windows (version 1.01s).

Results
Table I presents selected lifestyle characteristics of the 831 case patients (544 non-Hispanic Whites, 142 African-Americans, 105 Hispanic-Americans and 40 Asian-Americans) and the 755 control subjects (570 non-Hispanic Whites, 97 African-Americans, 68 Hispanic-Americans and 20 Asian-Americans) included in this study. The mean age at diagnosis was 65.5 years (SD = 5.63) for the case patients compared with 64.6 years (SD = 6.67) for control subjects at their assigned reference age. Relevant covariates considered simultaneously in the multivariable logistic regression analyses are shown in Table I. Risk of colon cancer was not significantly associated with type of menopause, age at menopause, age at menarche, physical activity or alcohol use. In contrast, risk of colon cancer decreased with increasing parity (P trend = 0.07), with increasing years of...
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Table I. Demographic and other characteristics of colon cancer patients and controls

<table>
<thead>
<tr>
<th>Race/ethnicity</th>
<th>Number of cases</th>
<th>Number of controls</th>
<th>Adjusted ORa</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-Hispanic White</td>
<td>544</td>
<td>570</td>
<td>1.00</td>
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</tr>
<tr>
<td>Black</td>
<td>142</td>
<td>97</td>
<td>0.76</td>
<td>0.56–1.11</td>
</tr>
<tr>
<td>Hispanic</td>
<td>105</td>
<td>68</td>
<td>0.91</td>
<td>0.70–1.18</td>
</tr>
<tr>
<td>Asian</td>
<td>40</td>
<td>20</td>
<td>0.76</td>
<td>0.56–1.02</td>
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</tbody>
</table>

Age at menopause (years)

<table>
<thead>
<tr>
<th>Age at menopause</th>
<th>Number of cases</th>
<th>Number of controls</th>
<th>Adjusted OR</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;60</td>
<td>191</td>
<td>224</td>
<td>1.00</td>
<td></td>
</tr>
<tr>
<td>61–65</td>
<td>199</td>
<td>182</td>
<td>0.85</td>
<td>0.64–1.13</td>
</tr>
<tr>
<td>66–70</td>
<td>240</td>
<td>182</td>
<td>0.83</td>
<td>0.61–1.12</td>
</tr>
<tr>
<td>&gt;70</td>
<td>201</td>
<td>167</td>
<td>0.76</td>
<td>0.57–1.01</td>
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</table>

Menopause type

<table>
<thead>
<tr>
<th>Menopause type</th>
<th>Number of cases</th>
<th>Number of controls</th>
<th>Adjusted OR</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Natural menopause</td>
<td>438</td>
<td>388</td>
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<tr>
<td>Complete hysterectomy</td>
<td>111</td>
<td>114</td>
<td>0.79</td>
<td>0.57–1.08</td>
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<tr>
<td>Simply hysterectomy</td>
<td>173</td>
<td>148</td>
<td>0.85</td>
<td>0.59–1.20</td>
</tr>
<tr>
<td>HT/other</td>
<td>92</td>
<td>93</td>
<td>1.10</td>
<td>0.78–1.57</td>
</tr>
<tr>
<td>Other</td>
<td>17</td>
<td>12</td>
<td>1.34</td>
<td>0.66–2.67</td>
</tr>
</tbody>
</table>

Age at menopause (years)

<table>
<thead>
<tr>
<th>Age at menopause</th>
<th>Number of cases</th>
<th>Number of controls</th>
<th>Adjusted OR</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;40</td>
<td>164</td>
<td>139</td>
<td>1.00</td>
<td></td>
</tr>
<tr>
<td>40–44</td>
<td>125</td>
<td>106</td>
<td>1.03</td>
<td>0.72–1.54</td>
</tr>
<tr>
<td>45–49</td>
<td>207</td>
<td>224</td>
<td>0.76</td>
<td>0.52–1.10</td>
</tr>
<tr>
<td>50–54</td>
<td>256</td>
<td>218</td>
<td>1.05</td>
<td>0.72–1.56</td>
</tr>
<tr>
<td>&gt;55</td>
<td>79</td>
<td>68</td>
<td>0.93</td>
<td>0.57–1.50</td>
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Age at menopause (years)

<table>
<thead>
<tr>
<th>Age at menopause</th>
<th>Number of cases</th>
<th>Number of controls</th>
<th>Adjusted OR</th>
<th>95% CI</th>
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<tr>
<td>&lt;11</td>
<td>183</td>
<td>179</td>
<td>1.00</td>
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<tr>
<td>12</td>
<td>210</td>
<td>160</td>
<td>1.34</td>
<td>1.01–1.88</td>
</tr>
<tr>
<td>13</td>
<td>207</td>
<td>230</td>
<td>0.94</td>
<td>0.72–1.30</td>
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<tr>
<td>14</td>
<td>102</td>
<td>95</td>
<td>1.09</td>
<td>0.77–1.62</td>
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Sex

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<tr>
<th>Number of cases</th>
<th>Number of controls</th>
<th>Adjusted OR</th>
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<tbody>
<tr>
<td>2</td>
<td>104</td>
<td>95</td>
<td>0.82</td>
</tr>
<tr>
<td>3</td>
<td>226</td>
<td>204</td>
<td>0.91</td>
</tr>
<tr>
<td>4</td>
<td>177</td>
<td>165</td>
<td>0.79</td>
</tr>
<tr>
<td>5</td>
<td>98</td>
<td>101</td>
<td>0.66</td>
</tr>
<tr>
<td>6</td>
<td>120</td>
<td>96</td>
<td>0.73</td>
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Family history of colorectal cancer

<table>
<thead>
<tr>
<th>Number of cases</th>
<th>Number of controls</th>
<th>Adjusted OR</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>No</td>
<td>717</td>
<td>671</td>
<td>1.00</td>
</tr>
<tr>
<td>Yes</td>
<td>114</td>
<td>84</td>
<td>1.23</td>
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</table>

OCS

<table>
<thead>
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<th>Number of cases</th>
<th>Number of controls</th>
<th>Adjusted OR</th>
<th>95% CI</th>
</tr>
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<tr>
<td>Never</td>
<td>551</td>
<td>460</td>
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<tr>
<td>&lt;2 years</td>
<td>110</td>
<td>80</td>
<td>1.14</td>
</tr>
<tr>
<td>2–5 years</td>
<td>57</td>
<td>60</td>
<td>0.82</td>
</tr>
<tr>
<td>&gt;5–14 years</td>
<td>47</td>
<td>83</td>
<td>0.42</td>
</tr>
<tr>
<td>&gt;14 years</td>
<td>66</td>
<td>72</td>
<td>0.80</td>
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Physical activity (h/week)

<table>
<thead>
<tr>
<th>Physical activity (h/week)</th>
<th>Number of cases</th>
<th>Number of controls</th>
<th>Adjusted OR</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Never</td>
<td>219</td>
<td>176</td>
<td>1.00</td>
<td></td>
</tr>
<tr>
<td>&gt;0.75</td>
<td>224</td>
<td>209</td>
<td>0.88</td>
<td>0.67–1.19</td>
</tr>
<tr>
<td>&gt;0.75–1.5</td>
<td>99</td>
<td>117</td>
<td>0.73</td>
<td>0.52–1.06</td>
</tr>
<tr>
<td>&gt;1.5–3</td>
<td>142</td>
<td>109</td>
<td>1.26</td>
<td>0.90–1.81</td>
</tr>
<tr>
<td>&gt;3</td>
<td>147</td>
<td>144</td>
<td>1.00</td>
<td>0.74–1.43</td>
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Years of smoking

<table>
<thead>
<tr>
<th>Years of smoking</th>
<th>Number of cases</th>
<th>Number of controls</th>
<th>Adjusted OR</th>
<th>95% CI</th>
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<tbody>
<tr>
<td>0</td>
<td>382</td>
<td>375</td>
<td>1.00</td>
<td></td>
</tr>
<tr>
<td>1–20</td>
<td>139</td>
<td>142</td>
<td>1.07</td>
<td>0.80–1.45</td>
</tr>
<tr>
<td>21–40</td>
<td>202</td>
<td>165</td>
<td>1.26</td>
<td>0.97–1.67</td>
</tr>
<tr>
<td>&gt;40</td>
<td>108</td>
<td>73</td>
<td>1.49</td>
<td>1.02–2.12</td>
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Table I. Continued

<table>
<thead>
<tr>
<th>Number of cases</th>
<th>Number of controls</th>
<th>Adjusted OR</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Years of alcohol use</td>
<td></td>
<td>0</td>
<td>1.00</td>
</tr>
<tr>
<td></td>
<td>21–40 381</td>
<td>165</td>
<td>0.76–1.19</td>
</tr>
<tr>
<td></td>
<td>&gt;40 67</td>
<td>65</td>
<td>0.59–1.29</td>
</tr>
</tbody>
</table>
| Current BMI (kg/m²)
|                  | <23.2 200          | 208          | 1.00         |
|                  | >23.2–26.2 193     | 191          | 1.11         | 0.81–1.47 |
|                  | >26.2–30.3 219     | 181          | 1.25         | 0.94–1.72 |
|                  | >30.3 219          | 175          | 1.15         | 0.92–1.71 |

*ORs were adjusted for age (<60, 61–65, 66–70 and >70 years), race/ethnicity (non-Hispanic White, African-American, Hispanic-American and Asian-American), education (high school or less, some college, college graduate and more than college), current BMI and simultaneously for all the other variables shown.

†Age 50 BMI and age 60 BMI were adjusted for in separate models replacing current BMI in the model.

‡The association between current HT and colon cancer risk was examined separately by select tumor characteristics (tumor stage and subsite of colon cancer), demographic factors (age at diagnosis, race/ethnicity and education), menopausal-related factors (e.g. type of menopause and age at menopause) as well as lifestyle-related factors (e.g. BMI, smoking, use of OC, use of aspirin and physical activity) that may potentially modify the HT–colon cancer association (Table III). The pattern of risk reduction in association with current ET use and current EPT use was observed consistently in these subgroup analyses, although the results were statistically significant in only some subgroups.

DNA methylation patterns in the colonic tissues of a subset of colon cancer patients were evaluated. Information on DNA
methylated in the normal colonic tissues was available on 234 colon cancer patients (164 never/ex-HT users and 70 current HT users; Table IV). On the basis that current HT users had lower risk of colon cancer, we treated ‘current HT users’ as ‘exposed’ and ‘never/ex-HT users’ as ‘non-exposed’ in these analyses. Suggestive differences in normal colonic DNA methylation levels for PGR and MGMT were found between current HT users and never/ex-HT users. Low DNA methylation patterns at PGR (P trend = 0.055) and high DNA methylation patterns at MGMT (P trend = 0.025) were associated with non-current HT use. Summary methylation scores for ESR1, ESR2 and PGR and for all seven genes combined were not significantly associated with current HT use. When we repeated the analysis using 0, −5–50 and >50 PMR cutoffs, results remained essentially the same with a slight strengthening in the association with PGR (P trend = 0.017) and a slight weakening in the association with MGMT (P trend = 0.062; see Table IV).

We also investigated DNA methylation patterns in the tumorous colonic tissues of 280 colon cancer patients (198 never/ex-HT users and 82 current HT users; Table V). Low DNA methylation level of ESR1 (P trend = 0.056) was more common in non-current HT users than in current HT users; this result was borderline statistically significant. When we investigated HT use and DNA methylation in tumorous colonic tissues using a summary methylation score for ESR1, ESR2 and PGR, HT use was inversely associated with this score; the adjusted ORs were 1.0, 0.92 and 0.49, respectively, in relation to scores of ≤1, 2 and 3 (P trend = 0.047). DNA methylation at all seven genes was not associated with current use of HT. When we repeated the analysis using 0, >0–5, >5–50 and >50 PMR cutoffs, results remained the same; the associations with ESR1 (P trend = 0.049) and the summary score for ESR1, ESR2 and PGR were essentially unchanged (P trend = 0.056; see Table V).

Discussion

The main objective of this population-based case–control study was to further investigate the role of different formulations of menopausal hormone and risk of colon cancer. A secondary objective was to explore the role of DNA methylation as a possible mechanism in explaining the effects of HT on risk. Although our DNA methylation analyses were conducted in only a subset of the case patients, we have identified some provocative clues that are worthwhile of further investigation (see below). Our study represents one of the few population-based epidemiological studies on HT and colorectal cancer that has included as part of the design of our study to explore how use of HT may influence colorectal cancer risk.

Results from this population-based case–control study suggest that current users of HT, ET or EPT experienced a significant reduced risk of colon cancer, but former users did not show similar benefit. Specifically, results from our study showed a risk reduction of 17% per 5 years of ET use and 12% per 5 years of EPT use. In a 1999 meta-analysis of 18 epidemiological studies on HT and colorectal cancer risk, ever hormone users experienced a 20% reduction in risk, whereas current hormone users displayed a 34% reduction in risk (1). Although formulation was not investigated in this meta-analysis, it is assumed that almost all the HT use was EPT since combined EPT was not commonly used during the 1970s and 1980s when most of these studies were conducted.

Since 1999, at least 10 studies (5 case–control, 4 prospective cohort and 1 controlled clinical trial) have investigated colorectal cancer risk patterns by formulations of HT. Use of ET and EPT was inversely associated with colorectal cancer risk in all five case–control studies (20–24), although statistically significant relations with ET (20,21) and EPT (22,24) were found only in some studies. Results from the observational prospective cohort studies are more mixed. Colorectal cancer risk was reduced among ET users (RR = 0.83, 95% CI = 0.70–0.99)
Table III. Use of menopausal hormones (per 5 years of use) and risk of colon cancer

<table>
<thead>
<tr>
<th>Number of cases/number of controls</th>
<th>Per 5 years of current ET use Adjusted OR* (95% CI)</th>
<th>Per 5 years of current EPT use Adjusted OR* (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumor stage</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Localized/regional</td>
<td>382</td>
<td>0.81 (0.72–0.92)</td>
</tr>
<tr>
<td>Metastatic</td>
<td>442</td>
<td>0.83 (0.75–0.93)</td>
</tr>
<tr>
<td>Subsite</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Right</td>
<td>450</td>
<td>0.81 (0.73–0.90)</td>
</tr>
<tr>
<td>Non-right</td>
<td>376</td>
<td>0.84 (0.75–0.95)</td>
</tr>
<tr>
<td>Age at diagnosis (years)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;65</td>
<td>388/405</td>
<td>0.71 (0.61–0.84)</td>
</tr>
<tr>
<td>&gt;65</td>
<td>438/348</td>
<td>0.83 (0.71–0.98)</td>
</tr>
<tr>
<td>Race/ethnicity</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Whites</td>
<td>542/569</td>
<td>0.83 (0.75–0.91)</td>
</tr>
<tr>
<td>Non-Whites</td>
<td>284/184</td>
<td>0.78 (0.64–0.95)</td>
</tr>
<tr>
<td>Education</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Less than or equal to high school</td>
<td>347/217</td>
<td>0.80 (0.70–0.91)</td>
</tr>
<tr>
<td>College</td>
<td>479/536</td>
<td>0.82 (0.73–0.93)</td>
</tr>
<tr>
<td>Menopause type</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Natural/HT</td>
<td>529/479</td>
<td>0.83 (0.69–1.00)</td>
</tr>
<tr>
<td>Surgical</td>
<td>279/262</td>
<td>0.83 (0.75–0.92)</td>
</tr>
<tr>
<td>Age at natural menopause (years)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;40</td>
<td>180/175</td>
<td>1.23 (0.72–2.10)</td>
</tr>
<tr>
<td>50+</td>
<td>255/211</td>
<td>0.50 (0.29–0.86)</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤26.26</td>
<td>392/398</td>
<td>0.82 (0.73–0.92)</td>
</tr>
<tr>
<td>&gt;26.26</td>
<td>434/355</td>
<td>0.85 (0.74–0.98)</td>
</tr>
<tr>
<td>Smoking</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-smoker</td>
<td>380/373</td>
<td>0.81 (0.71–0.92)</td>
</tr>
<tr>
<td>Ex/current smoker</td>
<td>446/380</td>
<td>0.84 (0.75–0.95)</td>
</tr>
<tr>
<td>OC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-user</td>
<td>451/353</td>
<td>0.83 (0.74–0.94)</td>
</tr>
<tr>
<td>Ex/current user</td>
<td>375/400</td>
<td>0.82 (0.71–0.94)</td>
</tr>
<tr>
<td>Aspirin user</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-user</td>
<td>550/460</td>
<td>0.75 (0.69–0.85)</td>
</tr>
<tr>
<td>Ex/current user</td>
<td>276/293</td>
<td>0.93 (0.81–1.07)</td>
</tr>
<tr>
<td>Physical activity</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>215/176</td>
<td>0.78 (0.63–0.95)</td>
</tr>
<tr>
<td>Yes</td>
<td>611/577</td>
<td>0.85 (0.77–0.94)</td>
</tr>
<tr>
<td>Alcohol use</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-drinker</td>
<td>438/378</td>
<td>0.82 (0.73–0.93)</td>
</tr>
<tr>
<td>Ex/current drinker</td>
<td>388/375</td>
<td>0.82 (0.72–0.93)</td>
</tr>
</tbody>
</table>

*OR adjusted for age (<60, 61–65, 66–70 and 70+ years), race/ethnicity (Caucasian, African-American, Latina and Asian), education (less than high school, high school, college and college+), type of menopause, age at menopause (<40, 40–44, 45–49, 50–54 and 55+ years), screening tests, BMI (<23.2, >23.2–26.2, >26.2–30.3 and >30.3), smoking years (never, 1–20, 21–40, and >40), alcohol years (never, 1–20, 21–40 and >40), parity (0, 1, 2, 3, 4 and 5+), family history of colon cancer (no/yes), years of OCs use (continuous), years of aspirin use (continuous) and years of physical activity (continuous).

and EPT users (RR = 0.78, 95% CI = 0.60–1.02) in the US Breast Cancer Detection Demonstration Project follow-up study (25). Similarly, in the Lombardy Italian linkage study, risk was reduced among EPT users (P trend = 0.04), which accounted for >90% of ET use in this study (results were not presented separately for ET and EPT use) (26). In the UK General Practice Research Database follow-up study, colorectal cancer risk was reduced among EPT users (RR = 0.56, 95% CI = 0.35–0.87) (27) but not among ET users (RR = 1.18, 95% CI = 0.72–1.92) (28). In contrast, risk of colorectal cancer did not differ significantly between ET users (RR = 0.83, 95% CI = 0.53–1.20) and EPT users (RR = 1.15, 95% CI = 0.74–1.79) compared with non-users in the WHI observation study (29). These results from the WHI observational study (29) differed from those reported in the WHI clinical trial in which there was a 37% reduction in risk (HR = 0.63, 95% CI = 0.43–0.92) associated with EPT use (0.625 mg/day of conjugated estrogen, 2.5 mg/day medroxyprogesterone) (20) but no significant effects of ET use (RR = 0.93, 95% CI = 0.75–1.15) (2).

Reasons for the differences in results by HT formulations are not apparent but age, body size, use of aspirin, physical activity and family history have been suggested as potential modifiers of the ET (EPT)–colorectal cancer association. For example, in the WHI clinical trial study, the ET–risk association differed significantly by age; ET use was associated with an increased risk (RR = 2.09, 95% CI = 1.08–4.04) in older (70–79 years of age) but not in younger women (RR = 0.88 and 0.59, respectively, for 60–69 and 50–59 years old) (2). However, in a case–control study conducted in Seattle, WA, USA, a significant beneficial effect of HT use was found in older women (70 years or older), whereas younger women (50–59 years old) did not show any reduction in risk (24). An effect of HT only among non-users of aspirin (22,30) and in physically inactive women (22) has also been reported. Although we found no evidence of significant modifying effects by age at diagnosis, body size, use of aspirin or other parameters in our study (Table III), continued monitoring of population trends of changing prevalence of risk factors (including body size, use of a non-steroidal anti-inflammatory drug and physical activity) and its potential effects on the magnitude and significance of HT–colorectal cancer associations may help to clarify differences in results between studies.

In addition, studies that are designed to explore the mechanisms by which menopausal hormone protects against colon cancer are needed. Several hypotheses have been proposed to explain the protective effects of exogenous estrogens against colorectal cancer. One proposed mechanism is that estrogens can interfere with bile acid metabolism. Bile acids are thought to initiate or promote malignant changes in the colon.
Another suggested mechanism is that menopausal estrogens might affect of estrogen that is mediated through ESR2 metabolism, decreasing secondary bile acid production (31). An effect of estrogen that is mediated through ESR2 expression is abundant, whereas ESR1 expression is uncommon in the colon (32). Another suggested mechanism is that menopausal estrogens might protect colon cancer by preventing the DNA methylation process of the ESR gene (7). Issa et al. (7) evaluated the methylation status of the ESR CpG island in both colorectal carcinomas and adenomatous polyps and found complete methylation of the ESR CpG in all 45 colorectal tumors analyzed, including early adenomas. Interestingly, they found less but still significant ESR1 CpG methylation in normal colon mucosa, with the methylation level increasing with age and from proximal to distal colon. Given that hypermethylation, and thus reduced expression, of the ESR in the colon is common with increasing age and that colon tumors typically arise in cells that have lost expression, of the ESR1 gene (7). Issa et al. (7) evaluated the methylation status of the ESR CpG island in both colorectal carcinomas and adenomatous polyps and found complete methylation of the ESR CpG in all 45 colorectal tumors analyzed, including early adenomas. Interestingly, they found less but still significant ESR1 CpG methylation in normal colon mucosa, with the methylation level increasing with age and from proximal to distal colon. Given that hypermethylation, and thus reduced expression, of the ESR in the colon is common with increasing age and that colon tumors typically arise in cells that have lost ESR expression, it is plausible that exposure to estrogens in menopausal hormones would help to maintain ESR gene expression and prevent ESR CpG island hypermethylation, thereby reducing the number of predisposed cells.

Table IV. Association between DNA methylation in ‘normal’ colonic tissues by current use of HT

<table>
<thead>
<tr>
<th>Gene</th>
<th>Non-current HT use (non-exposed)</th>
<th>Current HT use (exposed)</th>
<th>Adjusted ORb</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>ESR1</td>
<td>Low (&lt;10)</td>
<td>57</td>
<td>1.00</td>
<td>0.79–1.30</td>
</tr>
<tr>
<td></td>
<td>Middle (10–20)</td>
<td>29</td>
<td>0.72</td>
<td>0.50–1.00</td>
</tr>
<tr>
<td></td>
<td>High (20–30)</td>
<td>83</td>
<td>0.99</td>
<td>0.73–1.31</td>
</tr>
<tr>
<td></td>
<td>P trend</td>
<td></td>
<td>0.95</td>
<td></td>
</tr>
<tr>
<td>ESR2</td>
<td>No (0)</td>
<td>72</td>
<td>28</td>
<td>1.00</td>
</tr>
<tr>
<td></td>
<td>Yes (&gt;0)</td>
<td>89</td>
<td>42</td>
<td>0.94</td>
</tr>
<tr>
<td></td>
<td>P trend</td>
<td></td>
<td>0.85</td>
<td></td>
</tr>
<tr>
<td>PGRa</td>
<td>Low (&lt;5)</td>
<td>59</td>
<td>21</td>
<td>1.00</td>
</tr>
<tr>
<td></td>
<td>Middle (5–10)</td>
<td>60</td>
<td>20</td>
<td>0.55</td>
</tr>
<tr>
<td></td>
<td>High (10–20)</td>
<td>45</td>
<td>26</td>
<td>0.47</td>
</tr>
<tr>
<td></td>
<td>P trend</td>
<td></td>
<td>0.78</td>
<td></td>
</tr>
<tr>
<td>MGMTc</td>
<td>Low (&lt;5)</td>
<td>47</td>
<td>19</td>
<td>1.00</td>
</tr>
<tr>
<td></td>
<td>Middle (5–25)</td>
<td>56</td>
<td>38</td>
<td>1.04</td>
</tr>
<tr>
<td></td>
<td>High (25–50)</td>
<td>53</td>
<td>11</td>
<td>3.13</td>
</tr>
<tr>
<td></td>
<td>P trend</td>
<td></td>
<td>0.72</td>
<td></td>
</tr>
<tr>
<td>MYOD1</td>
<td>Low (&lt;10)</td>
<td>50</td>
<td>18</td>
<td>1.00</td>
</tr>
<tr>
<td></td>
<td>Middle (10–25)</td>
<td>32</td>
<td>17</td>
<td>0.75</td>
</tr>
<tr>
<td></td>
<td>High (25–50)</td>
<td>83</td>
<td>35</td>
<td>0.85</td>
</tr>
<tr>
<td></td>
<td>P trend</td>
<td></td>
<td>0.72</td>
<td></td>
</tr>
<tr>
<td>MLH1</td>
<td>Low (&lt;02)</td>
<td>52</td>
<td>17</td>
<td>1.00</td>
</tr>
<tr>
<td></td>
<td>Middle (02–05)</td>
<td>35</td>
<td>24</td>
<td>0.48</td>
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<tr>
<td></td>
<td>High (&gt;5)</td>
<td>65</td>
<td>28</td>
<td>0.76</td>
</tr>
<tr>
<td></td>
<td>P trend</td>
<td></td>
<td>0.14</td>
<td></td>
</tr>
<tr>
<td>Total score (ESR1, ESR2 and PGR)</td>
<td>Low (&lt;1)</td>
<td>61</td>
<td>19</td>
<td>1.00</td>
</tr>
<tr>
<td></td>
<td>Middle (2)</td>
<td>56</td>
<td>26</td>
<td>0.64</td>
</tr>
<tr>
<td></td>
<td>High (3)</td>
<td>41</td>
<td>25</td>
<td>0.51</td>
</tr>
<tr>
<td></td>
<td>P trend</td>
<td></td>
<td>0.14</td>
<td></td>
</tr>
<tr>
<td>Total score (all seven genes)</td>
<td>Low (&lt;3)</td>
<td>40</td>
<td>14</td>
<td>1.00</td>
</tr>
<tr>
<td></td>
<td>Middle (4–5)</td>
<td>53</td>
<td>23</td>
<td>1.46</td>
</tr>
<tr>
<td></td>
<td>High (&gt;5)</td>
<td>55</td>
<td>31</td>
<td>0.63</td>
</tr>
<tr>
<td></td>
<td>P trend</td>
<td></td>
<td>0.22</td>
<td></td>
</tr>
</tbody>
</table>

*aAdjusted for age, race/ethnicity, batch run and subsite (right/left) of colon.
*bAdjusted OR 1.0, 0.57 and 0.07 (P trend = 0.017) when we used cutpoints of 0–5, 5–50 and >50 PMR in the analysis.
*cAdjusted OR 1.0, 1.25 and 10.6 (P trend = 0.062) when we used cutpoints of 0–5, 5–50 and >50 PMR in the analysis.

Table V. Association between DNA methylation in ‘tumorous’ tissues by current use of HT

<table>
<thead>
<tr>
<th>Gene</th>
<th>Non-current HT use (non-exposed)</th>
<th>Current HT use (exposed)</th>
<th>Adjusted ORb</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>ESR1</td>
<td>Low (&lt;50)</td>
<td>57</td>
<td>16</td>
<td>1.00</td>
</tr>
<tr>
<td></td>
<td>Middle (50–100)</td>
<td>63</td>
<td>34</td>
<td>0.52</td>
</tr>
<tr>
<td></td>
<td>High (100+)</td>
<td>78</td>
<td>32</td>
<td>0.43</td>
</tr>
<tr>
<td></td>
<td>P trend</td>
<td></td>
<td>0.95</td>
<td></td>
</tr>
<tr>
<td>ESR2</td>
<td>Low (&lt;02)</td>
<td>58</td>
<td>21</td>
<td>1.00</td>
</tr>
<tr>
<td></td>
<td>Middle (02–05)</td>
<td>85</td>
<td>41</td>
<td>0.87</td>
</tr>
<tr>
<td></td>
<td>High (&gt;05)</td>
<td>43</td>
<td>20</td>
<td>0.79</td>
</tr>
<tr>
<td></td>
<td>P trend</td>
<td></td>
<td>0.57</td>
<td></td>
</tr>
<tr>
<td>PGRa</td>
<td>Low (&lt;3)</td>
<td>77</td>
<td>27</td>
<td>1.00</td>
</tr>
<tr>
<td></td>
<td>Middle (3–12)</td>
<td>50</td>
<td>19</td>
<td>0.72</td>
</tr>
<tr>
<td></td>
<td>High (12+)</td>
<td>70</td>
<td>35</td>
<td>0.54</td>
</tr>
<tr>
<td></td>
<td>P trend</td>
<td></td>
<td>0.57</td>
<td></td>
</tr>
<tr>
<td>MGMTc</td>
<td>Low (&lt;5)</td>
<td>41</td>
<td>19</td>
<td>1.00</td>
</tr>
<tr>
<td></td>
<td>Middle (5–50)</td>
<td>93</td>
<td>34</td>
<td>1.70</td>
</tr>
<tr>
<td></td>
<td>High (50+)</td>
<td>57</td>
<td>26</td>
<td>1.10</td>
</tr>
<tr>
<td></td>
<td>P trend</td>
<td></td>
<td>0.90</td>
<td></td>
</tr>
<tr>
<td>MYOD1</td>
<td>Low (&lt;50)</td>
<td>51</td>
<td>23</td>
<td>1.00</td>
</tr>
<tr>
<td></td>
<td>Middle (50–100)</td>
<td>54</td>
<td>24</td>
<td>0.99</td>
</tr>
<tr>
<td></td>
<td>High (100+)</td>
<td>93</td>
<td>32</td>
<td>1.13</td>
</tr>
<tr>
<td></td>
<td>P trend</td>
<td></td>
<td>0.72</td>
<td></td>
</tr>
<tr>
<td>MLH1</td>
<td>Low (&lt;1)</td>
<td>92</td>
<td>36</td>
<td>1.00</td>
</tr>
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<td></td>
<td>Middle (1–50)</td>
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<td>29</td>
<td>0.88</td>
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<tr>
<td></td>
<td>High (50+)</td>
<td>43</td>
<td>17</td>
<td>1.23</td>
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<td></td>
<td>P trend</td>
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<td>0.73</td>
<td></td>
</tr>
<tr>
<td>Total score (ESR1, ESR2 and PGR)</td>
<td>Low (&lt;1)</td>
<td>56</td>
<td>18</td>
<td>1.00</td>
</tr>
<tr>
<td></td>
<td>Middle (2)</td>
<td>60</td>
<td>22</td>
<td>0.92</td>
</tr>
<tr>
<td></td>
<td>High (3)</td>
<td>78</td>
<td>41</td>
<td>0.49</td>
</tr>
<tr>
<td></td>
<td>P trend</td>
<td></td>
<td>0.047</td>
<td></td>
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<td>Total score (all seven genes)</td>
<td>Low (&lt;3)</td>
<td>40</td>
<td>13</td>
<td>1.00</td>
</tr>
<tr>
<td></td>
<td>Middle (4–5)</td>
<td>60</td>
<td>24</td>
<td>0.69</td>
</tr>
<tr>
<td></td>
<td>High (&gt;5)</td>
<td>83</td>
<td>38</td>
<td>0.54</td>
</tr>
<tr>
<td></td>
<td>P trend</td>
<td></td>
<td>0.17</td>
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</table>

*aAdjusted for age, race/ethnicity, batch run and subsite (right/left) of colon.
*bAdjusted OR 1.0 and 0.49 (P trend = 0.049) when we used cutpoints of ≤50 and >50 PMR in the analysis.
*cAnalyses were conducted among those with data on all three genes: 194 non/ex-HT users and 91 current HT users had data in tumorous tissues. Adjusted OR 1.00, 0.82 and 0.47 (P trend = 0.056) when we used cutpoints of 0, 1 and 2+ using the new cutpoints of 0–5, >5–50 and >50 for ESR1, ESR2 and PGR, respectively, in the PMR analysis.
*dAnalyses were conducted among those with data on all seven genes: 183 non/ex-HT users and 75 current HT users with data in tumorous tissues.
However, contrary to our hypothesis that HT users would have reduced methylation, colon cancer patients who were current HT users tended to have higher DNA methylation at *ESR1*, *ESR2* and *PGR* than those who were non-current HT users; results were borderline statistically significant for *ESR1* in the tumorous colonic tissues (Table V) and for *PGR* in normal colonic tissues (Table IV). Current HT users and non-current HT users differed in a summary score that was constructed to capture DNA methylation of these genes (*ESR1*, *ESR2* and *PGR*) in tumorous colonic tissues (Table V). Few previous studies have investigated the relationship between HT use and DNA methylation. Woodson *et al.* (33) studied a subgroup of enrollees in the PolyPhen Prevention Trial, including 58 women who were taking HT at the baseline visit and 59 women who never took hormones. *ESR1* methylation status in baseline adenomas did not differ according to HT use but *ESR1* methylation status showed a strong inverse association with recurrence of advanced adenomas. Although *PGR* methylation was also investigated in this study, the authors noted that the prevalence of *PGR* methylation was too low to statistically evaluate in association with recurrence. Thus, our results and those of Woodson *et al.* (33) suggest no ‘deleterious effects’ of extensive methylation of *ESR1* and related genes on risk. Our finding of higher methylation of *MGMT* in the normal colonic tissues of non-current HT users is intriguing. Methylation of promoter *MGMT* is common and present in about half of colorectal tissues (34). Causes of *MGMT* promoter methylation are not well understood. Colorectal cancer patients with low folate and high alcohol intake have been found to have higher *MGMT* promoter methylation than those with high folate and low alcohol intake (35). Although we are not aware of previous studies on the effects of exogenous hormones on *MGMT* methylation, there is suggestion that common *MGMT* polymorphisms may influence the risk of colorectal cancer in women and that the effect may be modified by postmenopausal hormone use (36).

Our study represents a population-based epidemiological study that has been designed specifically to investigate the associations between different regimens of HT and we were able to adjust carefully for various potential confounders in our analyses. We also evaluated whether HT use is associated with DNA methylation of several genes of particular interest. Despite these strengths, a limitation is that our overall response rate was modest. The most common reasons we failed to interview a potentially eligible case was that the subject was too ill or deceased. We attempted to assess whether the risk patterns observed in association with ET and EPT differed by stage of cancer since those with earlier stage cancers at diagnosis were more probably to be interviewed. Our results were generally similar for earlier stage (i.e. localized to regional) and more advanced stage colon cancers and by various demographic and risk factor subgroups (Table III). Although our exploratory findings on DNA methylation are intriguing, this substudy was conducted in only approximately one-third of the total number of cases we interviewed. In addition, only a small number of genes were investigated. It is evident that better understanding of the mechanisms by which exogenous hormones may influence colon cancer risk is needed.

**Funding**

National Cancer Institute (CA17054 and 5P30 CA 014089-34); National Institute of Environmental Health Science (5P30 ES007048-13).

**Acknowledgements**

We are grateful to all the study participants for their contributions and support. We thank the entire data collection team. Incident colon cancer cases for this study were collected by the University of Southern California Cancer Surveillance Program, which is supported under subcontract by the California Department of Health. The Cancer Surveillance Program is also part of the National Cancer Institute’s Division of Cancer Prevention and Control Surveillance, Epidemiology and End Results Program under contract number N01CN25403.

**Conflict of Interest Statement:** None declared.

**References**


Received August 5, 2009; revised December 29, 2009; accepted January 6, 2010

Menopausal hormones, DNA methylation, colon cancer