

## Estrogen Stimulates the Expression of Mismatch Repair Gene *hMLH1* in Colonic Epithelial Cells

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### Abstract

Estrogen is reported to have a protective effect on colon cancer; however, the underlying mechanism is unclear. Impaired mismatch repair plays an important role in colonic carcinogenesis. The purpose of this study was to investigate the association of estrogen on regulating mismatch repair expression in colonic epithelial cells. In cultured COLO205 cells, the effect of estradiol (E2) and antagonist ICI182.780 on the expression of *hMLH1* and *hMSH2* was studied using reverse transcription-PCR and Western blotting. The correlation between serum level E2 and the expression of *hMLH1* and *hMSH2* in colonic mucosal tissue of 42 healthy individuals was also examined using reverse transcription-PCR and immunohistochemical staining. E2 increased the expression of *hMLH1* in COLO205 cells, which was suppressed by ICI182.780. However, the effect of E2 on *hMSH2* expression was not significant in COLO205 cells. In healthy individuals, a strong positive correlation of E2 level with *hMLH1* expression in normal colonic epithelial cell was observed when serum E2 level was >45 pg/mL, but no correlation was seen between E2 and *hMSH2* expression. E2 affects the expression of *hMLH1* but not *hMSH2* *in vitro*, and high serum E2 level correlates with *hMLH1* expression *in vivo*. These findings suggest that the anticolon cancer effect of estrogen may be related to *hMLH1* regulation. *Cancer Prev Res*; 3(8); 910–6. ©2010 AACR.

### Introduction

Age-adjusted colon cancer incidence is higher for men than for women (1). This finding even extends to colonic adenomas (2). The sex-specific difference in colon cancer and precancerous lesion has been attributed to estrogen, whereas large-scale population studies found that postmenopausal estrogen use significantly reduces the risk of colon cancer (3, 4). Although some have postulated that the effect of estrogen on cell proliferation and apoptosis may be the reason (5), more specific underlying mechanism of why estrogen has anticolon cancer effect but has opposite effect on other cancers (for example endometrial cancer) remains to be investigated.

The DNA mismatch repair (MMR) system consists of proteins that act in concert to recognize and coordinate the repair of nucleotide base mismatches and slippage mistakes at microsatellite sequences on newly synthesized DNA. It plays a key role in maintaining genomic stability. Patients with germline mutations of any of the four major MMR genes (*hMSH2*, *hMLH1*, *hMSH6*, or *hPMS2*) have Lynch syndrome (or hereditary nonpolyposis colorectal cancer), the most common familial form of colorectal cancer. Another 10% to 15% of sporadic colorectal cancers are caused by epigenetic silencing of the *hMLH1* gene. MMR dysfunction causes microsatellite instability (MSI). It has been reported that estrogen is associated with MSI, whereas the incidence of MSI-positive colon cancer is lower in young women than in older women, and postmenopausal estrogen replacement therapy can reduce the risk of MSI-positive colon cancer (6). These findings suggest that estrogen might prevent carcinogenesis by altering MMR function in colonic cells. It has been reported that estradiol (E2) can upregulate MMR activity in endometrial glandular cells (7). However, there has been no direct examination of the effects of estrogen on MMR gene expression in colonic epithelial cells. In this study, we investigated the effect of estrogen on the expression of two representative MMR genes (*hMLH1* and *hMSH2*) in cultured malignant colonic epithelial cells, and correlated the serum estrogen level with MMR gene expression in human colonic epithelial cells from 42 healthy individuals.

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doi: 10.1158/1940-6207.CAPR-09-0228

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## Materials and Methods

### Cell culture

COLO205 cells (an estrogen receptor  $\beta$ -positive colon cancer cell line, purchased from Cell Resource Center, Beijing Union Medical Center) were cultured in phenol red-free RPMI1640 medium containing 10% charcoal-filtered fetal bovine serum for 24 hours. The cells were then serum starved (0.1% charcoal-filtered fetal bovine serum) with thymine (2 mmol/L) for 20 hours. Subsequently, the medium was replaced with serum-starved medium containing various concentration of estradiol E2 (Sigma; ranges from  $0-1 \times 10^{-6}$  mol/L) with or without  $1 \times 10^{-5}$  mol/L estrogen receptor antagonist ICI182.780 (Sigma). After 48 hours of incubation, cells were harvested and used for reverse transcription-PCR (RT-PCR) and Western blotting.

### Semiquantitative RT-PCR and Western blotting

Total RNA of colonic mucosal tissue and cultured cells were, respectively, extracted with Trizol (Invitrogen). RT-PCR was carried out according to the method described by Morimoto et al. (8). Primers used in this study were as follows: *hMLH1*, sense, 5'-GTGCTGGCAATCAAGG-GACCC-3', anti-sense, 5'-CACGGTTGAGGCATTGGG-TAG-3'; *hMSH2*, sense, 5'-GTCGGCTTCGTGCGCTTCTT-3', anti-sense, 5'-TCTCTGGCCATCAACTGCGGA-3'; and  $\beta$ -actin, sense, 5'-ACACTGTGCCCATCTACGAGG-3', anti-sense, 5'-AGGGGCCGACTCGTCATACT-3'.

Western blotting was done using total proteins extracted from cultured cells and the antibodies for *hMLH1* and *hMSH2* (both from Proteintech Group, Inc.) and glyceraldehyde-3-phosphate dehydrogenase (Kangche BIO-TECH) according to the method described by Shiozawa et al. (9).

Both Western blotting and RT-PCR were done twice. The bands detected in RT-PCR and Western blotting were analyzed using densitometry with the TotalLab 2.0 software. To evaluate the relative levels of expression of the target genes, the value of the internal standard ( $\beta$ -actin and *GAPDH*, respectively) was used as baseline gene expression.

### Quantitative RT-PCR

We used quantitative RT-PCR to confirm the results of semiquantitative RT-PCR in COLO205 cells. PCR was done in a volume of 20  $\mu$ L containing 12.5  $\mu$ L  $2 \times$  PCR Mix (BioChain), 2  $\mu$ L cDNA, 0.3  $\mu$ L EvaGreen (Biotium), 0.5  $\mu$ L of primers each, and 4.2  $\mu$ L DEPC-treated water. The mixtures were amplified by an initial denaturation step of 95°C for 5 minutes; 45 cycles of denaturation at 95°C for 30 seconds, annealing at 60°C for 30 seconds, and extension at 72°C for 30 seconds; and a final elongation step at 72°C for 5 minutes. Three repetitions were done for each sample. The relative levels of expression of the target genes mRNA were calculated by  $\Delta\Delta$ CT-method relative to the internal standard  $\beta$ -actin and control lane ( $E_2 = 0$ ). Primers used in this study were as follows: *hMLH1*, sense, 5'-TTCGTGGCAGGGGTATTTCG-3', anti-

sense, 5'-GCCTCCCTCTTTAACAATCACTT-3'; *hMSH2*, sense, 5'-GCTGGAATAAGGCATCCAAGG-3', anti-sense, 5'-CACCAATGGAAGCTGACATATCA-3'; and  $\beta$ -actin, sense, 5'-CATGTACGTTGCTATCCAGGC-3', anti-sense, 5'-CTCCTTAATGTCACGCACGAT-3'.

### Healthy subjects

A total of 42 healthy individuals underwent colonoscopy, including 23 females and 19 males who were enrolled in the study. The female subjects included women with a normal menstrual cycle ( $n = 9$ ), perimenopausal ( $n = 5$ ), and postmenopausal women ( $n = 9$ ). Subjects who had taken estrogen replacement therapy were excluded from the study. Blood was taken half an hour before colonoscopy for determining the serum E2 level. Under colonoscopic examination, four or five biopsies of normal colonic mucosal tissue were taken in the right hemicolon. Two fresh tissue samples were used to extract total RNA for semiquantitative RT-PCR; the remaining tissues were fixed with formalin and then embedded in paraffin for immunohistochemical staining. All of these samples were histologically confirmed normal. The study was approved by the Beijing Military General Hospital's ethical committee.

### Immunohistochemistry

Immunohistochemical staining was carried out according to the method described previously by Sheng et al. (10) with the exception that different primary antibodies were used for *hMLH1* (Proteintech Group, Inc.), *hMSH2* (Proteintech Group, Inc.), and proliferating cell nuclear antigen (PCNA; Zhongshan Goldenbrige Biotechnology Co., Ltd.) in colonic mucosal tissue. For negative control, the primary antibodies were replaced with mouse IgG (Dakopatts). For each marker, a positivity index (PI) was calculated by multiplying the intensity of nuclear staining [strongly positive (3), moderately positive (2), weakly positive (1), or negative (0)] with the percentage of stained cells. All immunohistochemical samples were independently evaluated by two pathologists (Jin and Sheng), and the mean of two results was used as the final PI value.

### Statistical analyses

ANOVA was used in the correlations of E2 and ICI182.780 of different concentrations with the expression of *hMLH1* and *hMSH2* in cultured cells. Student's *t* test was used in the comparisons of *hMLH1* and *hMSH2* expression in colonic mucosal tissue between groups divided according to age or gender. Due to the nonlinear relationship, curve estimation modeling was first carried out in the correlation analyses between serum E2 level and the expression of *hMLH1* and *hMSH2* in colonic mucosal tissue (PI values of immunohistochemical staining and semiquantitative RT-PCR results). The mode with adjusted  $R^2$  value closest to 1 was regarded as the best fitting model, and then E2 level at the turning point was used as threshold to separate low ( $<45$  pg/mL) and high ( $\geq 45$  pg/mL) E2 levels, whereas the association of E2 and expression was

analyzed using the Spearman's rank correlation. The SPSS 13.0 software was used for statistical analyses. A strong correlation was defined if the Spearman's  $\rho$  is  $>0.4$  or  $<-0.4$ . All statistical tests were done two sided, and  $P < 0.05$  was considered statistically significant.

## Results

### Effect of E2 on the expression of *hMLH1* and *hMSH2* *in vitro*

As shown in Fig. 1, RT-PCR and Western blotting showed that E2 increased the expression of *hMLH1* in COLO205 cells in mRNA and protein levels. E2-induced upregulation was suppressed by the estrogen antagonist ICI182.780. The upregulation effect of E2 on *hMLH1* expression on both mRNA and protein level was dose dependent between  $1 \times 10^{-12}$  and  $1 \times 10^{-8}$  mol/L (about 0.2725 to 2,725 pg/mL; Fig. 1D). However, no effect of E2 was seen for *hMSH2* expression (Fig. 1).

### Correlation between the expression of MMR in human colonic mucosa epithelial cells and serum E2 levels

A group of 42 healthy individuals were recruited to study the relationship of serum E2 level and the expression of MMR (Table 1). Of the 42 individuals, 23 were female (age ranged from 20-70 y, and mean age was 50 y) and 19 were male (age ranged from 22-72 y, and mean age was 46 y). The serum E2 levels were between 0 and 182.3 pg/mL with a mean of 48.5 pg/mL in 23 female subjects, whereas in the 19 male subjects, the serum E2 levels were between 0 and 75.2 pg/mL with a mean of 24.9 pg/mL. In seven subjects, serum E2 levels were too low ( $<5.0$  pg/mL) to be determined. There were no significant differences in mRNA expression and protein PI values of *hMLH1* and *hMSH2* between subjects older than 50 years ( $n = 20$ ) and younger than 50 years ( $n = 22$ ), and also, there were no significant differences in *hMLH1* and *hMSH2* expression between male and female subjects ( $P > 0.05$  for both by Student *t* test), suggesting that age and gender had no significant effects on the expression of *hMLH1* and *hMSH2*.

An "S"-shaped distribution curve was observed for the serum E2 level in relation to *hMLH1* mRNA and protein expression in colonic mucosa epithelial cells (Fig. 2). Curved estimation showed that cubic equation model was the best model to analyze the correlations of E2 level with *hMLH1* mRNA expression and PI value (adjusted  $R^2$  were 0.362 and 0.406;  $P$  were 0.002 and 0.000, respectively; Fig. 2). Furthermore, the stratified analysis showed that this model was more significant in the female subjects (adjusted  $R^2$  were 0.642 and 0.694;  $P$  were 0.000 and 0.000, respectively; data not shown) but not suitable for the male ( $P > 0.05$  for both). There was no curve model suitable for the correlations of E2 with *hMSH2* mRNA expression and PI value ( $P > 0.05$ ; Fig. 2). Using the turning point of the curve (45 pg/mL) as the threshold, subjects were divided into low E2 ( $<45$  pg/mL) group and high E2 ( $\geq 45$  pg/mL) group. In the high E2 group, a strong positive correlation

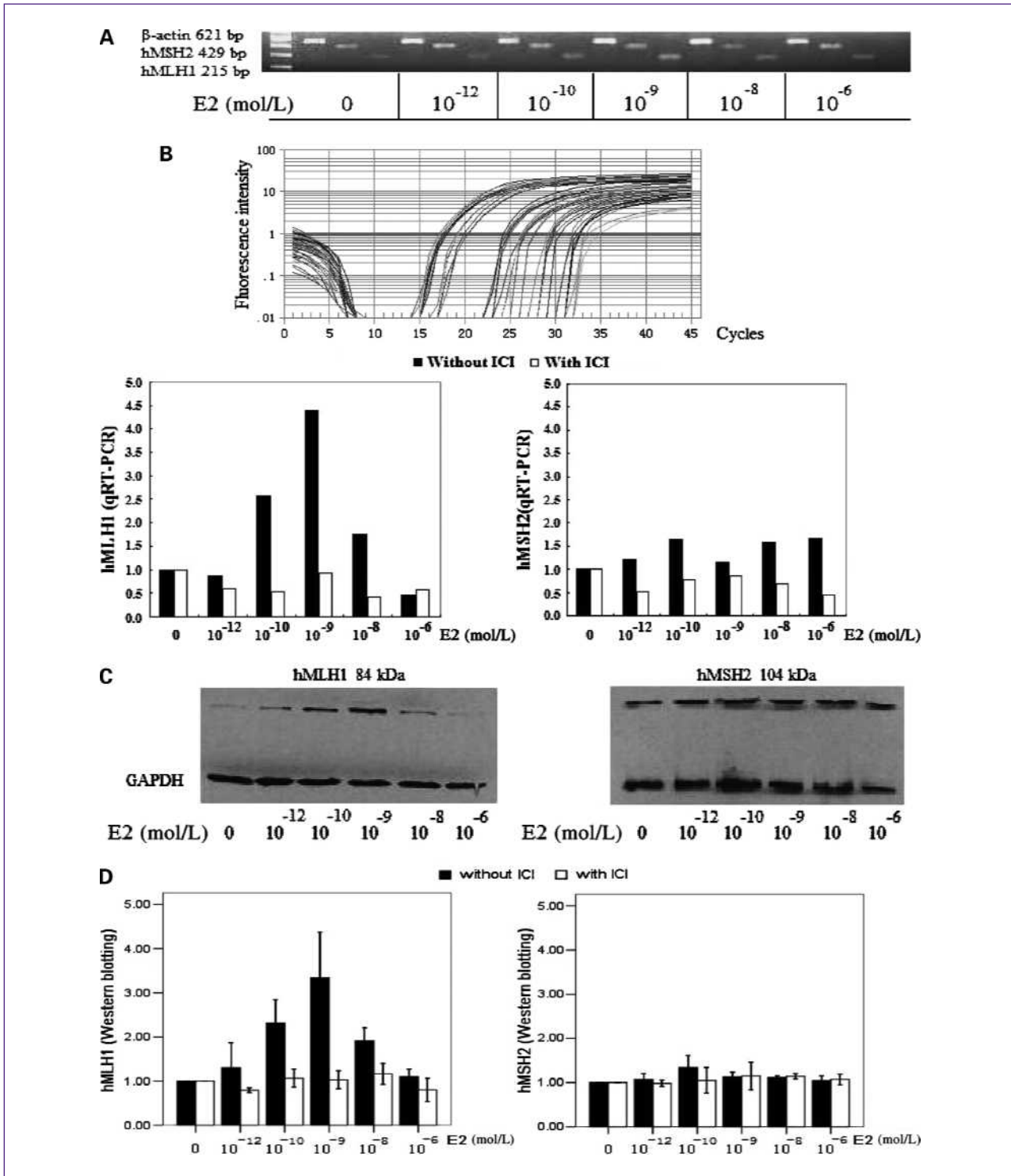
of E2 level with *hMLH1* expression at both mRNA and protein levels (mRNA:  $P = 0.003$ ,  $\rho = 0.701$ ; protein PI value:  $P = 0.000$ ,  $\rho = 0.874$ ) were observed, whereas positive correlation between E2 level and *hMSH2* protein value ( $P = 0.016$ ,  $\rho = 0.592$ ) and no correlation between E2 level and *hMSH2* mRNA expression ( $P = 0.122$ ,  $\rho = 0.403$ ) were seen. At the E2 level less than 45 pg/mL, the expression of either *hMLH1* or *hMSH2* was not significantly correlated with serum E2 level ( $P > 0.05$ ). The PI of PCNA was inversely correlated with serum E2 level ( $P = 0.006$ ,  $\rho = -0.431$ ; data not shown).

Figure 3 showed representative images of immunohistochemical staining of *hMLH1*, *hMSH2*, and PCNA in colonic mucosa epithelial cells from women with high and low E2 level (A and B, respectively). Comparing to mucosa from low E2 level, the staining was much more intense in *hMLH1* and *hMSH2* in mucosa cells from women with high expression of E2. In contrast, the PCNA level of staining was more intense in mucosa cells from low E2 but less intense in mucosa cells from high E2 level.

## Discussion

It has been reported that in hereditary nonpolyposis colorectal cancer families, the incidence of colorectal cancer is higher in male mutation carriers than in female (11), suggesting that estrogen is likely to have protective effects on hereditary nonpolyposis colorectal cancer. Slattery et al. (6) found that postmenopausal estrogen replacement therapy could reduce the risk of MSI-positive colon cancer in women. These studies suggest that estrogen may prevent colorectal cancer caused by MMR dysfunction, which thus provided the foundation for our hypothesis that estrogen may affect the expression of DNA MMR gene(s). Our study therefore directly tested the effect of estrogen on MMR gene expression, first using a unique cell line COLO205. This cell line only has estrogen receptor  $\beta$  expression, but no estrogen receptor  $\alpha$  expression (12). Indeed, we observed a direct effect of E2 on the upregulation of *hMLH1* gene expression in mRNA and protein level, which is antagonized by the estrogen inhibitor ICI182.780. Furthermore, a strong correlation between serum E2 level and *hMLH1* mRNA and protein level in colonic epithelial cells in healthy individuals was observed. Our study suggested that the regulation of expression of *hMLH1* is probably at transcriptional level; this is in line with the finding that *hMLH1* promoter has a hemiestrogen responsive element (13). Interestingly, our study showed that E2 had no significant effects on *hMSH2* expression in colonic epithelial cells. Because MMR protein expression is strongly associated with the function of MMR (7), our finding offered an important mechanism to explain the anticancer effect of estrogen.

Our study in normal individuals showed that only when serum E2 levels were higher than 45 pg/mL, a strong positive correlation with *hMLH1* gene expression was observed. To our knowledge, this is the first report of the estrogen level correlation with MMR expression of colonic



**Fig. 1.** Results of RT-PCR and Western blotting in cultured cell. A, representative result of semiquantitative RT-PCR for *hMLH1* and *hMSH2* in E2-treated COLO205 cells. B, result of quantitative RT-PCR analysis in E2 and antagonist ICI182.780-treated COLO205 cells. Graphs indicate the levels of expression of the target genes mRNA relative to the internal standard  $\beta$ -actin and control lane (E2 = 0) measured by the  $\Delta\Delta C_T$ -factor. Solid columns, no inhibitor (ICI182.780); open columns, cells treated with inhibitor. C, representative result of Western blotting for *hMLH1* and *hMSH2* in E2-treated COLO205 cells. D, dose effect of E2 and antagonist ICI182.780 on COLO205 cells. Graphs indicate the levels of expression (columns, mean; bars, SD) of the target genes relative to the internal standard GAPDH and control lane (E2 = 0) measured for protein (Western blotting) by densitometry. Solid columns, no inhibitor (ICI182.780); open columns, cells treated with inhibitor. ANOVA showed that the expression of *hMLH1* protein was increased by treatment with E2 ( $F$  was 3.300, and  $P$  was 0.019) in COLO205 cells, and this upregulation could be suppressed by ICI182.780 ( $F$  was 14.559, and  $P$  was 0.001).

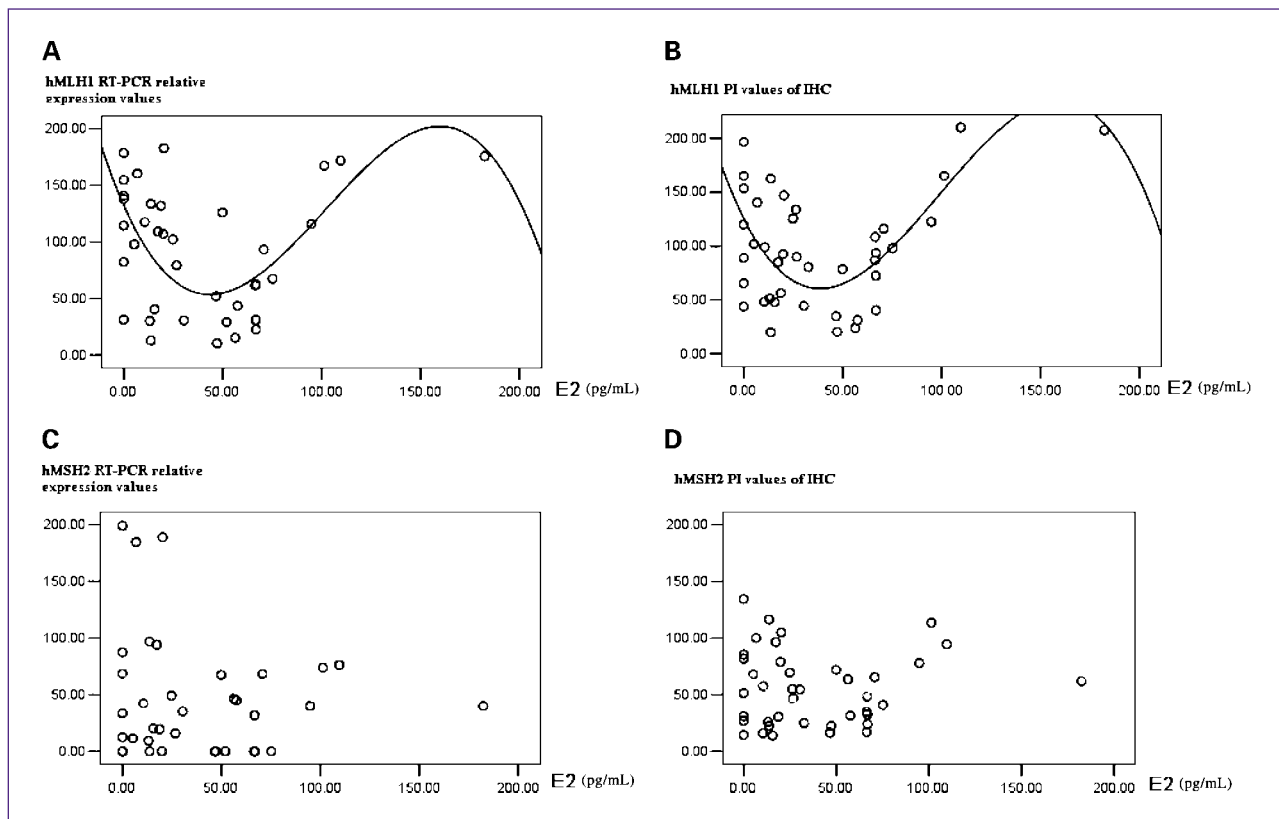
**Table 1.** E2 level and *hMLH1* and *hMSH2* mRNA, and immunohistochemical results stratified by gender and age in healthy subjects

Gender	Age	n	E2	mRNA		PI values of immunohistochemistry	
				<i>hMLH1</i>	<i>hMSH2</i>	<i>hMLH1</i>	<i>hMSH2</i>
Male	<50 y	12	32.8 ± 25.8	116.5 ± 51.4	74.7 ± 75.8	103.6 ± 36.7	59.6 ± 27.4
	≥50 y	7	11.4 ± 9.3	80.1 ± 40.3	56.9 ± 77.9	79.3 ± 40.9	52.6 ± 51.3
Female	<50 y	10	80.0 ± 45.2	95.9 ± 62.2	37.7 ± 30.1	116.1 ± 68.1	69.0 ± 27.6
	≤50 yd	13	24.2 ± 26.3	80.9 ± 56.0	26.4 ± 30.3	85.7 ± 56.8	43.4 ± 26.1

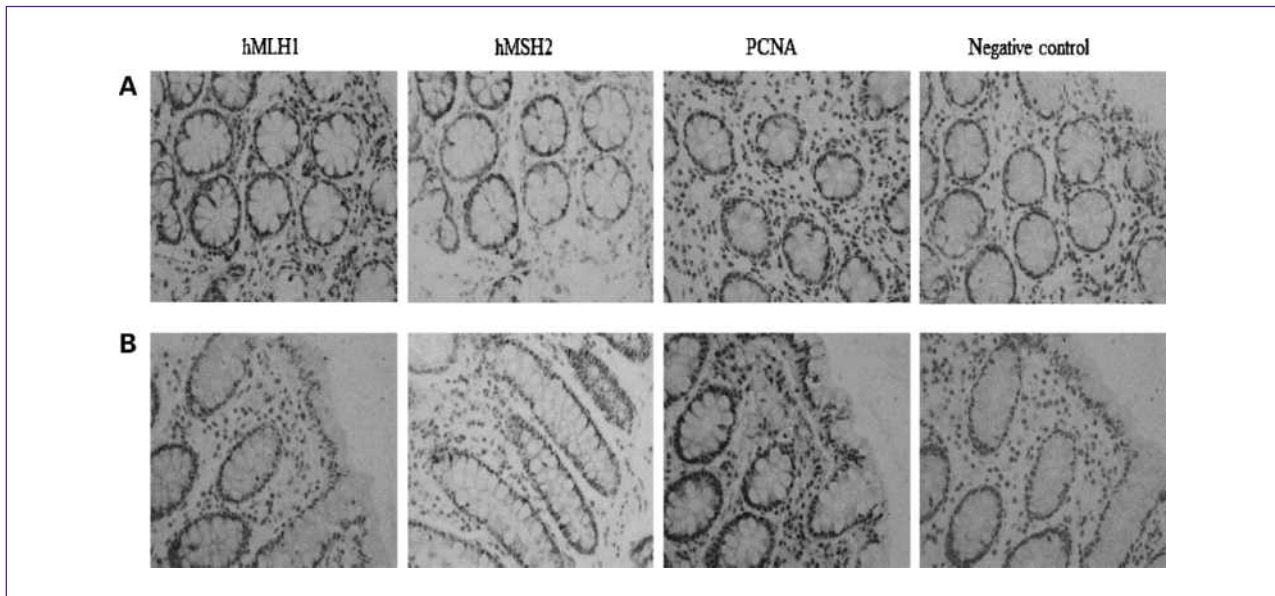
NOTE: All data were shown as mean ± SD.

cells in human. Interestingly, however, there was no significant correlation between *hMLH1* expression and serum E2 level when E2 level was lower (E2, <45 pg/mL). We speculate that when E2 level is low, the proliferation activity of colonic epithelial cells may override the effect of E2 on *hMLH1* expression. It has been reported that estrogen can inhibit colonic epithelial cell proliferation (14–16).

This is consistent with our observation that estrogen level correlated negatively with cell proliferation marker PCNA expression. When serum E2 level is low, the proliferation of colonic epithelial cells may increase, and the expression of *hMLH1* may be augmented in proliferating cells (17). Therefore, when E2 was decreased to a certain level, the effect of cell proliferation on *hMLH1* expression became



**Fig. 2.** Correlation between the expression of MMR and serum E2 levels *in vivo*. Curve estimation showed that cubic equation model was the best model that described the correlations of E2 level with *hMLH1* mRNA expression and immunohistochemical PI value. The PI was calculated by multiplying the intensity of nuclear staining [strongly positive (3), moderately positive (2), weakly positive (1), or negative (0)] with percentage of stained cells so the range was 0 to 300, which was shown on the Y-axis ordinate (B and D). There was no curve model suitable for the correlations of E2 with *hMSH2* mRNA expression and PI value.



**Fig. 3.** Results of immunohistochemistry staining. Representative images of immunohistochemistry staining (with 3,3'-diaminobenzidine coloring and hematoxylin counterstaining) for hMLH1, hMSH2, and PCNA in normal colonic mucosal tissue obtained from two women, one with high (A, E2: 109.6 pg/mL) and one with low (B, E2: 17.3 pg/mL) serum levels of E2. For negative control, the primary antibodies were replaced with mouse IgG. Photos were taken at  $\times 400$  magnification using an Olympus BX40 microscope.

more apparent, and *hMLH1* expression fails to decline even with the decrease of E2 level. This might also explain that whereas there was a significant difference in estrogen level between male and female subjects, there was no significant difference in *hMLH1* expression between males and females.

Similar to our study, Miyamoto et al.'s study (7) showed that estrogen could increase the expression of *hMLH1* and *hMSH2*, and enhances MMR activity in endometrial glandular cells. Whereas estrogen may be a protective factor for colon cancer, it is also considered a risk factor for endometrial cancer. Therefore, additional mechanisms must come into play in terms of the effect of estrogen on carcinogenesis in different organ systems. Regardless, however, the E2-induced upregulation of MMR expression may act as an intrinsic defense against colon carcinogenesis. Further studies will be needed to delineate the functional effect on MMR activity as the result of E2-induced *hMLH1* overexpression.

In summary, our study showed that E2 increases the expression of *hMLH1* *in vitro*, whereas serum E2 level, when

over 45 pg/mL, directly correlates with the expression of MMR in actual human colonic epithelial cells. Estrogen might prevent colon cancer by regulating MMR system, and the interplay between cell proliferation control and MMR dysfunction at low E2 level may lead to occurrence of colonic cancer.

#### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

#### Grant Support

National Natural Science Foundation of China, grant no. 30940086. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received 10/21/2009; revised 03/04/2010; accepted 03/25/2010; published OnlineFirst 07/27/2010.

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