Estradiol regulates miR-135b and mismatch repair gene expressions *via* estrogen receptor-β in colorectal cells

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Abstract

Estrogen has anti-colorectal cancer effects which are thought to be mediated by mismatch repair gene (MMR) activity. Estrogen receptor (ER) expression is associated with microRNA (miRNA) expression in ER-positive tumors. However, studies of direct link between estrogen (especially estradiol E$_2$), miRNA expression, and MMR in colorectal cancer (CRC) have not been done. In this study, we first evaluated the effects of estradiol (E$_2$) and its antagonist ICI182,780 on the expression of miRNAs (miR-31, miR-155 and miR-135b) using COLO205, SW480 and MCF-7 cell lines, followed by examining the association of tissue miRNA expression and serum E$_2$ levels using samples collected from 18 colorectal cancer patients. E$_2$ inhibited the expressions of miRNAs in COLO205 cells, which could be reversed by E$_2$ antagonist ICI 182.780. The expression of miR-135b was inversely correlated with serum E$_2$ level and ER-β mRNA expression in CRC patients' cancer tissues. There were significant correlations between serum E$_2$ level and expression of ER-β, miR-135b, and MMR in colon cancer tissue. This study suggests that the effects of estrogen on MMR function may be related to regulating miRNA expression *via* ER-β, which may be the basis for the anti-cancer effect in colorectal cells.

Keywords: colon neoplasms; DNA mismatch repair; estrogen receptor β; estrogen; microRNAs

Introduction

Colorectal cancer (CRC) is the fourth most common cause of death from cancer, affects around 1.2 million people world wide, with about 608,700 fatalities per year (Ferlay *et al.*, 2010). Both the prevalence (Ries *et al.*, 2000) and the lifetime risk of CRC (Froggatt *et al.*, 1999) are significantly lower in females than in males. This is particularly true when comparing pre-menopausal women to age-matched men. The sex-specific difference in CRC incidence has been attributed to estrogen (Chlebowski *et al.*, 2004). Estrogen exerts its effects mainly through two members of the estrogen receptor (ER) superfamily, ER-α and ER-β. ER-α expression is low in normal colonic mucosa and colon cancer cells, while ER-β is predominant in human colonic mucosal cells. ER-β is significantly decreased in colonic neoplasms compared to normal colonic mucosa. Decreased ER-β expression may lead to hyperproliferation, decreased differentiation, and decreased apoptosis of colonic epithelial cells (Maria and Paola, 2008), suggesting that ER-β is likely to be involved in the regulation of colonic tumor growth.

DNA mismatch repair (MMR) system plays an

important role in maintaining genomic stability. The MMR gene alteration including genetic and sporadic mutations, commonly occur in the early stages of the neoplastic process. MMR dysfunction can cause microsatellite instability (MSI). It has been reported that estrogen is associated with MSI (Slattery *et al.*, 2001). In our previous study, estradiol (E_2) has shown to up-regulate major MMR gene (*hMLH1*) activity in colonic epithelial cells (Jin *et al.*, 2010). These findings suggest that estrogen's anti-colorectal cancer effect may be mediated by MMR function in colonic cells. However, the pathway through which estrogen regulates MMR function is still not well understood.

MicroRNAs (miRNAs), a series of small noncoding RNAs of 19 to 22 nucleotides, are involved in many important cellular processes such as development, differentiation, proliferation, cell cycle progression, apoptosis, inflammation, and stress response (Lee and Dutta, 2009; Lu and Liston, 2009; Wang and Lee, 2009). Since expression alterations of some miRNAs are associated with a series of genetic events, their expression patterns may either have pro- or anti- cancer effects (He *et al.*, 2005; Johnson *et al.*, 2005; O'Donnell *et al.*, 2005). Several miRNAs expression alterations have been reported in CRC, including miR-135b, miR-96, miR-183, miR-133a, miR-133b, miR-21, miR-31, miR-145, miR-203, miR-223, miR-155, etc (Bandres *et al.*, 2006; Cummins *et al.*, 2006; Volinia *et al.*, 2006; Lanza *et al.*, 2007; Sarver *et al.*, 2009). However, the mechanism for miRNA change in CRC is not well understood. Recent studies have indicated that estrogens can regulate miRNAs expression in human breast cancer cells, endometrial stromal cells, and myometrial smooth muscle cells (Pan *et al.*, 2007; Wickramasinghe *et al.*, 2009). Therefore, we hypothesize that estrogen may have similar effects on miRNAs expression in colorectal epithelial cells.

In this study, we investigated the relationships of Estradiol, ER-β, and the expression of MMR genes (*hMLH1* and *hMSH2*) and miRNA in cultured human colon cancer cells (COLO205 and SW480), human breast cancer cells (MCF-7) and patient tissue/serum samples. Our findings suggest that estrogen, through ER-β, regulates miRNA and MMR expression.

Results

Apoptosis induced by E_2 in COLO205 and SW480

After treatment with different concentrations of E_2 for 48 h, the apoptotic cells (Annexin-V+/PI-) were significantly increased in COLO205 (high levels of

ER-β expression, and no ER-α expression cells) (Qiu *et al.*, 2002) with a dose-dependent manner between 1×10^{-6} and 1×10^{-10} M of E_2, and the effect was the strongest at 1×10^{-9} M of E_2 (Figure 1A) (*P* < 0.001). Estrogen receptor antagonist, ICI182,780, inhibited E_2-induced apoptosis in COLO205 cells (Figure 1B). However, the effect of E_2 on apoptotic induction was not seen in SW480 which has very low levels of ER-β expression cells (Figure 1C) (Hartman *et al.*, 2009). The finding shows that estrogen's apoptosis induction effect appears to be specifically in ER-β expression cells.

Effect of E_2 on the expression of miRNAs *in vitro*

The expression levels of miR-31, miR-155, miR-135b, miR-203 and miR-223 in COLO205 cells were then determined before and after the treatment by E_2, respectively. RT-qPCR results indicated that E_2 decreased the levels of miR-31, miR-155 and miR-135b in a time-dependent manner (Figure 2A). To determine if the reduction of these miRNAs by E_2 was mediated by estrogen receptor, COLO205 cells were pre-incubated with 100 nM estrogen antagonist ICI182,780 for 6 h, followed by E_2 incubation. Our results indicated that ICI182,780 reversed E_2-induced repression of miR-31, miR-155 and miR-135b (Figure 2B). E_2 did not however affect miRNA expression in either SW480 cells, which is known to have very low levels of ER-β, or MCF-7 cells, in which ER-α represents the largely predominant form, while ER-β is only barely detectable (Figures 2C and 2D) (Perillo *et al.*, 2000; Hartman *et al.*, 2009; Wilkins *et al.*, 2010). The findings suggest that the differential effect of E_2 on miRNA expression in different cell lines relates to the ER-β, or the ratio of ER-β to ER-α.

Effect of E_2 on the expression of ER-β, hMLH1 and hMSH2 *in vitro*

RT-qPCR and Western blotting showed that E_2 increased the expression of ER-β and *hMLH1*, but at less degree of *hMSH2*, at both mRNA and protein levels in COLO205 cells, in a time and dose-dependent manners (Figures 3A and 3D). These effects were again inhibited by ICI182,780 (Figure 3B). No changes in the expression of *hMLH1*, *hMSH2* and ER-β mRNA were seen in MCF-7 and SW480 cells (Figures 3C and 3D). The results suggest that E_2, *via* ER-β, inhibits miRNA and increases *hMLH1* expression in COLO205 cells.
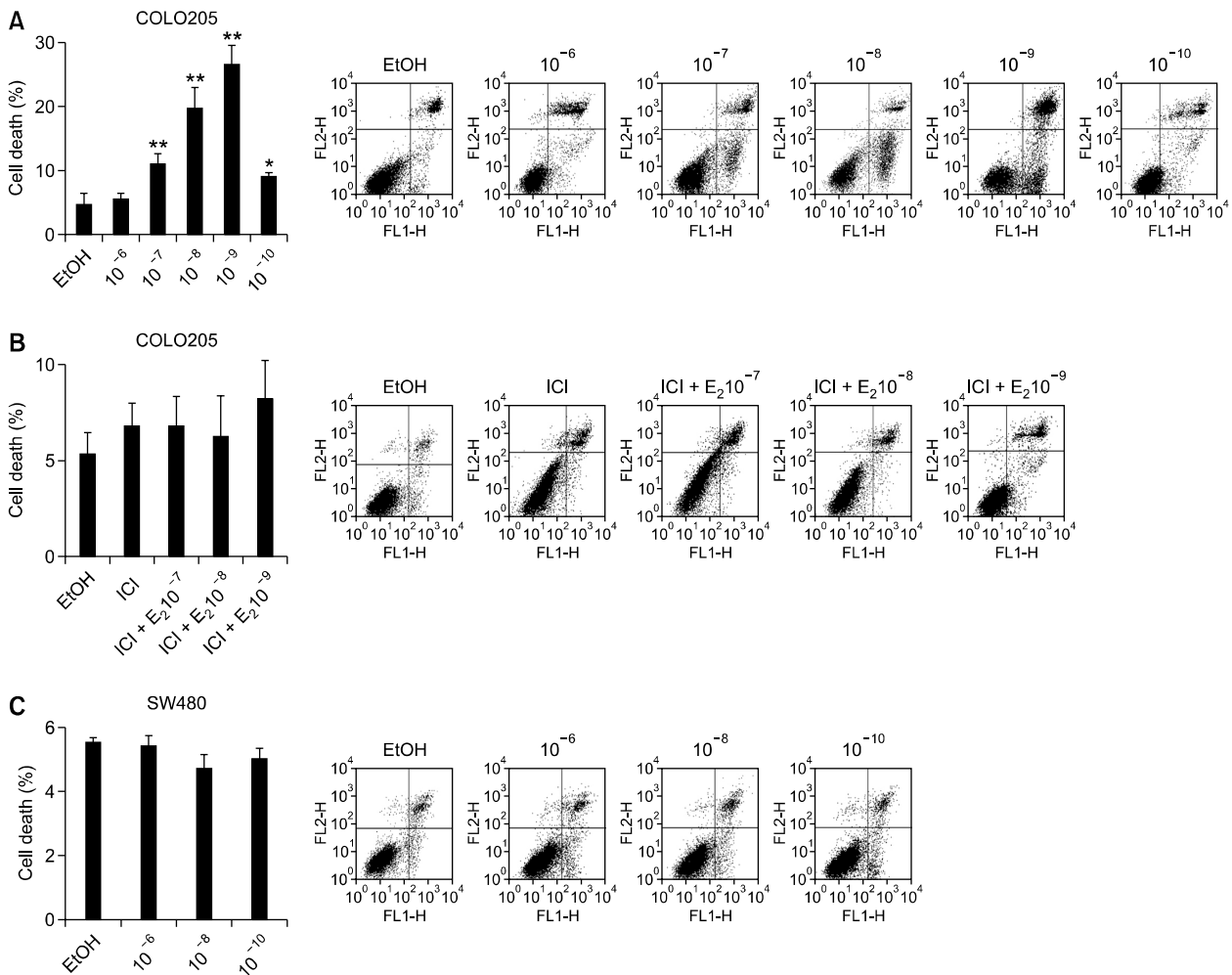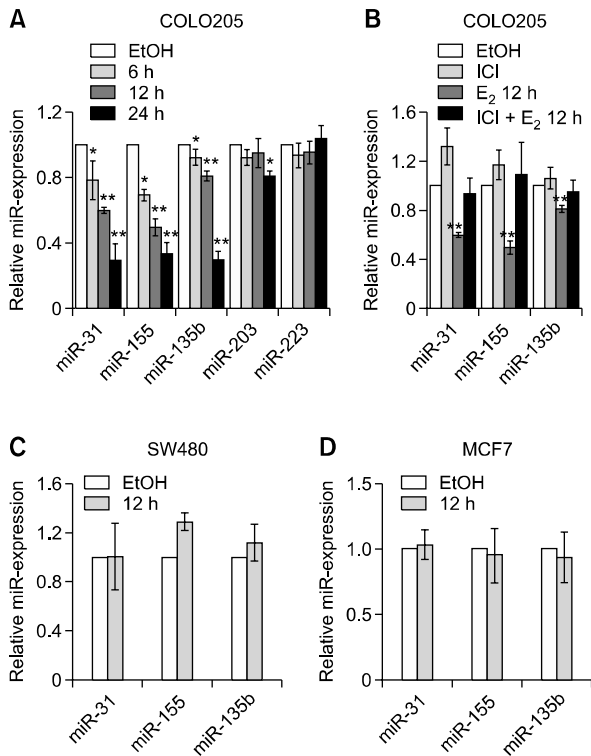
Figure 1. Effect of Estrogen (E_2) on apoptosis induction in COLO205 and SW480 cells. Apoptosis was determined by FITC-conjugated Annexin-V/PI assay kit (Abcam) and flow cytometry. COLO205 cells treated with 10^{-6}-10^{-10} M of E_2 (A) and estrogen antagonist, ICI182,780, prior to incubation with E_2 (B). SW480 cells treated with E_2 (C). The cells sorted at the Annexin-V+/PI- fraction (bottom right quadrant) were early apoptotic cells. Mean $\pm$ SD from 3 independent experiments.

Effect of E_2 on MMR and several miRs in ER-β over-expression cells

To confirm the regulation effects of E_2 on MMR and several miRs were mediated through *ERbeta*, we transfected ER-β plasma to up-regulate ER-β expression in SW480 cells. *ERbeta* protein expression was not up-regulated in vector transfected SW480 cells, whereas it was up-regulated in pRST7-ER-β transfected SW480 cells after 24 and 48 h of transfection by immunoblot (Figure 4A). RT-qPCR results indicated that E_2 decreased the levels of miR-31, miR-155 and miR-135b in pRST7-ER-β transfected SW480 cells, but not in vector cells (Figure 4B). RT-qPCR and western blotting showed that E_2 increased the expression of *hMLH1*, but at less degree of *hMSH2*, at both mRNA and protein levels in pRST7-ER-β transfected SW480 cells (Figures 4C and 4D).

Correlations of serum E_2 level with the expression of miRNA, ER-β, and MMR genes in human cancer tissue and adjacent non-cancer tissue

To extend the above *in vitro* findings to actual patient, a group of 18 CRC patients were recruited to study the relationships of serum E_2 level with miRNA, and mRNA expression of MMR and ER-β. Of the 18 patients, the serum E_2 levels were between 8.93 and 69.16 pg/ml with a mean of 28.08 pg/ml in the 9 male patients, while in the 9 female patients, the serum E_2 levels were between 24.09 and 70.18 pg/ml with a mean of 38.09 pg/ml. Both cancerous tissues and adjacent non-cancerous tissues were analyzed for four miRNAs (miR-135b, miR-31, miR-155, and miR-203) expression, and mRNA levels of ER-β, and MMR (*hMLH1* and *hMSH2*). In cancer tissue, ΔCt and $\Delta\Delta$Ct (non-cancerous tissue as control) values of miR-135b (R^2

Figure 2. Effect of Estrogen (E_2) on inhibiting the expression of miR-31, miR-135b and miR-155. Quantitative RT-PCR analysis indicates that E_2 induced the down-regulation of mature miR-31, miR-155, and miR-135b with time, but not miR-223 in COLO205 cells (A). ICI182,780 reversed E_2-induced inhibition of miR-31, miR-155 and miR-135b of COLO205 cells (B). SW480 (C) and MCF7 (D) cells were treated with EtOH and E_2 for 12 h, there were no significant changes in miR-31, miR-155 and miR-135b expression. Values presented were fold increase compared to EtOH for each miRNA and were calculated as described in 'Materials and Methods' section. Mean $\pm$ SD from 3 independent experiments. Significant differences between treated group versus EtOH control were calculated by ANOVA (*$P < 0.05$ and **$P < 0.01$).

= 0.716, P = 0.001; R^2 = 0.675, P = 0.002) (Figure 5A) and miR-31 (R^2 = 0.567, P = 0.014; R^2 = 0.561, P = 0.019) (Figure 5B) were significantly correlated with serum E_2 concentrations, but this was not seen for miR-155 and miR-203 (R^2 = 0.027, P = 0.515; R^2 = 0.035, P = 0.459) (Figure 5C, respectively).
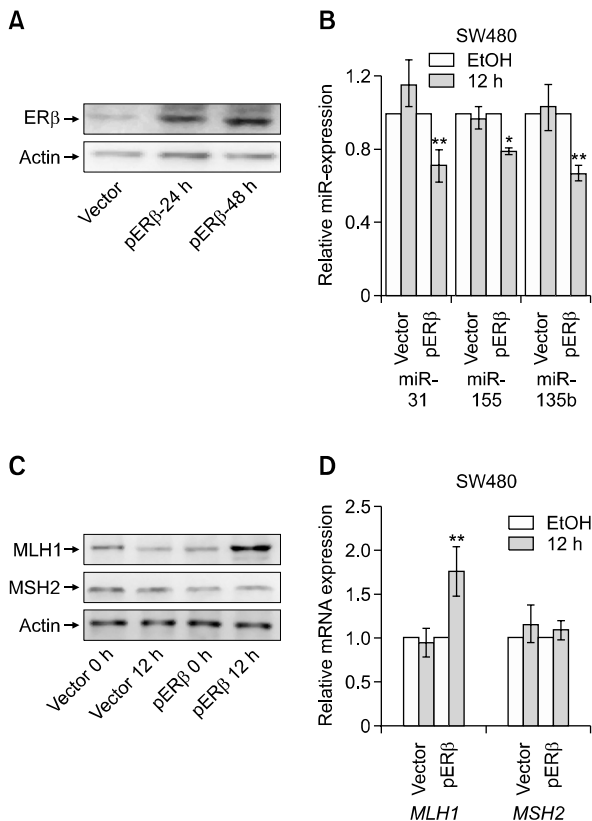
We also analyzed the correlations of E_2 level with ER-β mRNA in the 18 CRC patients. In cancer tissue, the ΔCt and $\Delta\Delta$Ct (tumor versus non-tumor) values of ER-β mRNA were positively correlated (R^2 = 0.583, P = 0.011; R^2 = 0.525, P = 0.025) (Figure 5D) with serum E_2 concentrations, but *hMLH1* and *hMSH2* (Figure not shown) mRNA were not correlated with serum E_2 concentrations.

Since miR-155 has been shown to inhibit *hMLH1* expression in cultured malignant colonic epithelial cells (24), we analyzed the correlation of miR-135b, miR-31 and miR-155 with *hMLH1* in the 18 CRC patients. In cancer tissue, the $\Delta\Delta$Ct values of
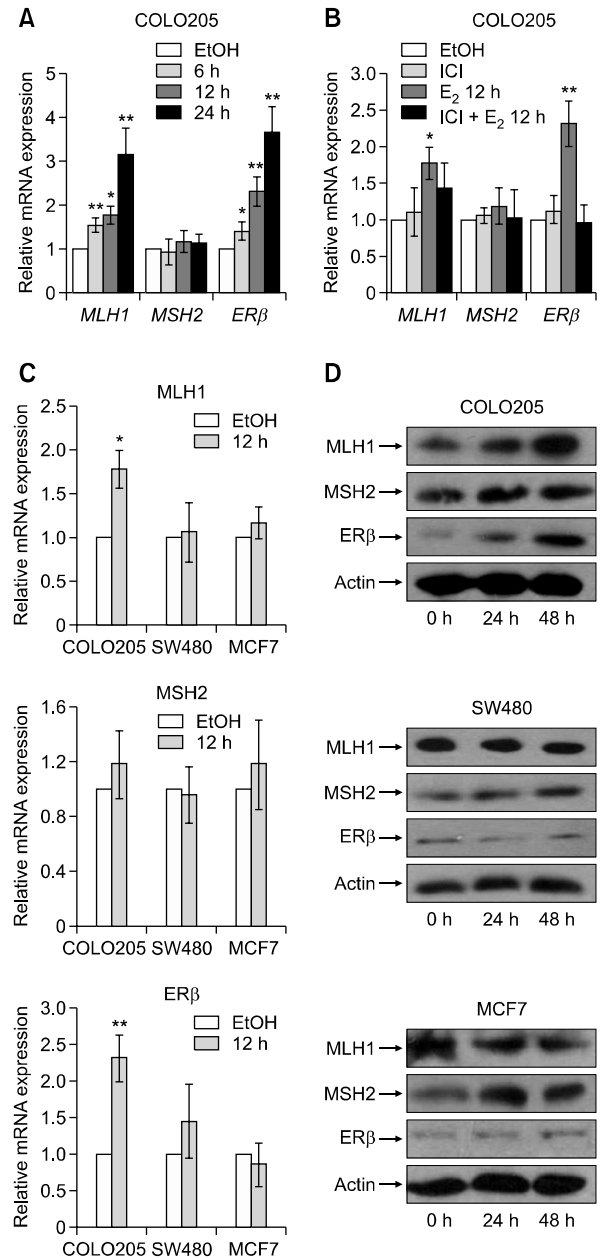
Figure 3. Effects of Estrogen (E_2) on the expression of *MLH1*, *MSH2* and ER-β mRNA. *MLH1* and ER-β mRNA determined by RT-qPCR in COLO205 cells (A) treated with EtOH or 10^{-9} M of E_2 for 0 h, 6 h, 12 h and 24 h. *MLH1* and ER-β mRNA determined by RT-qPCR in COLO205 cells (B) pre-treated with estrogen antagonist ICI 182.780 followed by EtOH or 10^{-9} M of E_2 for 0 h and 12 h. Effects of E_2 on *MLH1*, *MSH2*, and ER-β mRNA (C) measured by RT-qPCR in COLO205, SW480, and MCF7 cells. Cells were treated with EtOH, 10^{-10} and 10^{-9} M of E_2 for 12 h. Mean $\pm$ SD from 3 independent experiments. Significant differences between treated group versus EtOH control were calculated by ANOVA (*$P < 0.05$ and **$P < 0.01$). Effects of E_2 on protein level expression (D) of *MLH1*, *MSH2* and ER-β in COLO205, SW480, and MCF7 cells.

hMLH1 mRNA were negatively correlated with that of miR-135b and miR-155 (data not show). This was not seen for miR-31 (data not show). As *in vitro*

A

ERβ →
Actin →

Vector   pERβ-24 h   pERβ-48 h

B

SW480
☐ EtOH
▨ 12 h

Relative miR-expression

Vector / pERβ
miR-31

Vector / pERβ
miR-155

Vector / pERβ
miR-135b

C

MLH1 →
MSH2 →
Actin →

Vector 0 h   Vector 12 h   pERβ 0 h   pERβ 12 h

D

SW480
☐ EtOH
▨ 12 h

Relative mRNA expression

Vector / pERβ
MLH1

Vector / pERβ
MSH2

Figure 4. Effect of Estrogen (E_2) on MMR and several miRs in ER-β over-expression cells. ERbeta protein expression (A) was not up-regulated in vector transfected SW480 cells, whereas it was up-regulated in pRST7-ER-β transfected SW480 cells after 24 and 48 h of transfection. Quantitative RT-PCR analysis (B) indicates that E_2 induced the down-regulation of mature miR-31, miR-155, and miR-135b in SW480-ERbeta cells, but not in vector transfected SW480 cells. Effects of E_2 on *MLH1* and *MSH2* mRNA (C) measured by RT-qPCR in SW480- ERbeta and vector cells. Mean ± SD from 3 independent experiments. Significant differences between treated group versus EtOH control were calculated by ANOVA (*$P < 0.05$ and **$P < 0.01$). Effects of E_2 on protein level expression (D) of *MLH1* and *MSH2* in SW480- ERbeta and vector cells.

study cited above showed that E_2 down-regulates the expression of miR-31, miR-155 and miR-135b in COLO205 cells through ER-β, we analyzed the correlation of ER-β mRNA with the expression of three miRNAs in cancer tissue. Only the ΔΔCt value of miR-135b was correlated with the ΔΔCt values of ER-β mRNA (data not show).

Differential expression of miRNA and mRNA for ER-β and MLH1 in cancer versus adjacent non-cancer tissue from CRC patients

Finally, we analyzed the differential expression of miRNA in cancer versus adjacent non-cancer tissues. Interestingly, among the four tested miRNAs, miR-31, miR-155 and miR-135b ($P < 0.05$) were

exhibited higher expressions, while there were no changes in miR-203 expression in cancer tissue compared with adjacent non-cancerous tissue (Figure 5E). Separate analyses of CRC patients showed that the ΔCt ± SD of miR-31 was 11.2 ± 2.1 in cancer tissue and 12.7 ± 1.9 in cancer-surrounding tissue (ANOVA, $P = 0.032$), the ΔCt ± SD of miR-155 was 9.8 ± 1.6 in cancer tissue and 11.2 ± 1.9 in cancer-surrounding tissue (ANOVA, $P = 0.024$), and the ΔCt ± SD of miR-135b was 9.5 ± 2.3 in cancer tissue and 11.1 ± 1.9 in cancer-surrounding tissue (ANOVA, $P = 0.027$). The results also indicated that the up-regulation of miR-135b was the most marked in cancer tissue (Figure 5E). Thus, miR-135b may be a potential marker for colorectal cancer.

We also analyzed ER-β and *MLH1* expression in cancer and adjacent non-cancer tissue. ΔCt ± SD of ER-β mRNA was 10.2 ± 1.4 in non- cancerous tissue and 8.9 ± 1.9 in cancer tissue (ANOVA, $P = 0.009$), the ΔCt ± SD of *hMLH1* mRNA was 4.4 ± 1.5 in non-cancerous tissue and 3.8 ± 2.8 in cancer tissue (ANOVA, $P = 0.436$), the ΔCt ± SD of *hMSH2* mRNA was 2.3 ± 1.5 in non-cancerous tissue and 1.3 ± 0.9 in cancer tissue (ANOVA, $P = 0.086$) (Figure 5F). Only ER-β showed significant changes (down-regulation) in cancerous versus adjacent non-cancerous tissue (Figure 5F).

Discussion

This study revealed that E_2 increased *hMLH1* expression in COLO205 cells, which could be inhibited by Estrogen antagonist ICI182,780. Previously we found that in healthy individuals, a strong positive correlation of E_2 level with *hMLH1* expression in normal colonic epithelial cell was observed when serum E_2 level was > 45 pg/ml (Jin *et al.*, 2010). Recent studies have indicated that estrogens can regulate miRNA expression in human breast cancer cells, endometrial stromal cells, and myometrial smooth muscle cells (Pan *et al.*, 2007; Wickramasinghe *et al.*, 2009). Based on these results, we hypothesize that E_2 may regulate miRNA expression that may result in the regulation of *hMLH1* expression in colonic epithelial cells.

In this study, COLO205, SW480 and MCF-7 cells were used. We found that E_2 could induce apoptosis in ER-β expression COLO205 cells, but not in SW480 cells with very low levels of ER-β expression, and not in MCF-7 cells with high levels of ER-α expression. Further, in COLO205 cells E_2 down-regulated the expression of miR-31, miR-155 and miR-135b and up-regulated mRNA and protein expression of ER-β and *hMLH1*, which were
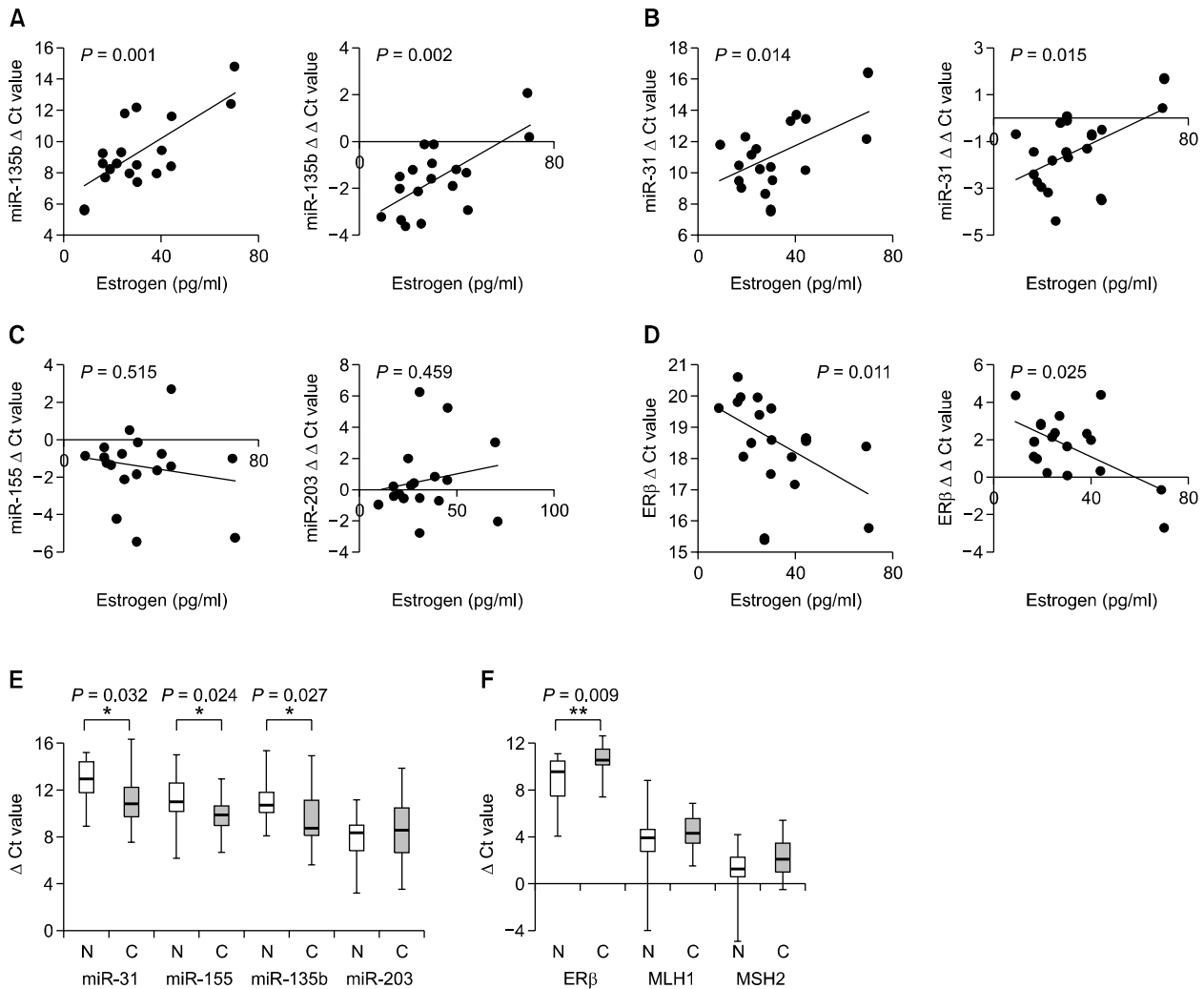
Figure 5. Correlations of serum Estrogen levels with miRNA and ER-β mRNA expression in CRC patients' tissues. Correlations of serum Estrogen levels with miRNA and ER-β mRNA expression in CRC patients' tissues. Serum Estrogen level was measured by ELISA assay whereas miRNA and ER-β mRNA was analyzed by RT-qPCR assay as described in Material and Methods. Correlation of serum estrogen concentrations with miR135b (A) in tumor ΔCt and ΔΔ Ct (tumor versus non-tumor). Correlation of serum estrogen concentrations with miR-31 (B) in tumor ΔCt and ΔΔ Ct. Correlation between the expression levels of miR-155 as well as miR-203 (C) in patients' cancer tissues and serum estrogen concentrations. Correlations of serum Estrogen concentrations with ER-β Mrna (D) in tumor ΔCt and ΔΔ Ct. Box plots with median, 25 th and 75 th percentiles, and range of expression levels of miR-31, miR-155, miR-135b, and miR-203 (E) in 18 tumor tissues relative to adjacent non-tumor tissue. Significant levels of differences were determined by ANOVA. Box plots with median, 25 th and 75 th percentiles, and range of expression levels of ER-β, *hMLH1*, *hMLH2*, (F) in 18 tumor tissues relative to adjacent non-tumor tissue. Significant levels of differences were determined by ANOVA (*$P < 0.05$ and **$P < 0.01$).

inhibited by ICI182,780. We also transfected the ER-β expressing plasmid to up-regulate ER-β expression in SW480 cells with very low levels of ER-β expression. In pRST7-ER-β transfected SW480 cells, E_2 down-regulated the expression of miR-31, miR-155 and miR-135b and up-regulate mRNA and protein expression of *hMLH* were also observed. Therefore, we hypothesize that E_2 may through ER-β to regulate MMR and miRNA expression. MiRNAs are reported to be able to down-regulate the core MMR heterodimer protein (Valeri *et al.*, 2010a, 2010b). Therefore, E_2 may

up-regulate MMR gene through down-regulation of miR-31, miR-155 and miR-135b. Further, E_2 appears to only up-regulate *hMLH1* expression without the effect on the other core MMR gene, *hMSH2*, in COLO205 and transfected SW480 cells. Based on the data above, we hypothesize that during CRC carcinogenesis, E_2 may through ER-β to inhibit miR-31, miR-155 and miR-135b expression, as a result leading to the up-regulation of MMR activity to prevent cancer progression (see Figure 6, hypothetical diagram).

To verify the relationship of E_2 and miRNA
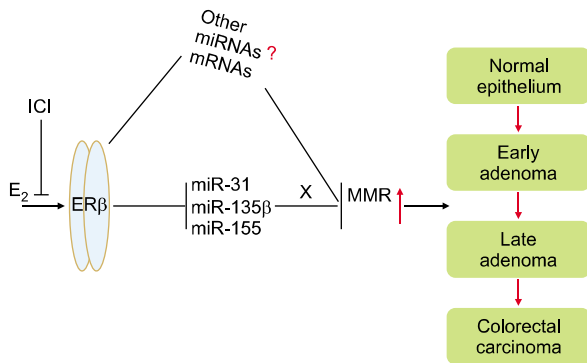
Figure 6. A simplified hypothetical diagram of interrelationships between Estrogen, ER-β, miRNA, and MMR. Estrogen regulates miRNA expression through binding to ER-β. The resulted decreased miRNA expression may affect MMR function that eventually prevents the transformation of normal colonic epithelium to cancer. ICI - estrogen inhibitor.

expression *in vivo*, adjacent non-cancerous tissue, cancer tissue and serum samples from 18 CRC patients were analyzed. The results indicated that serum E_2 levels were strongly and negatively correlated with miR-31 and miR-135b expression, but not for miR-155 expression in cancer tissue. We also found that in cancer tissue, *hMLH1* mRNA levels were negatively correlated with that of miR-155 and miR-135b expression, whereas ER-β mRNA levels were positively correlated with serum E_2 concentrations. Interestingly, only miR-135b has a significant correlation with serum E_2 level, ER-β and *hMLH1* expression. Thus, we hypothesize that during cancer progression, a decreased estrogen and/or reduced ER-β expression result in the increase of miR-135b, which contributes to MMR instability and eventually colorectal cancer carcinogenesis (Figure 6).

This study showed that there was no significant correlation between *hMLH1* expression and serum E_2 level in CRC patients. Our previous study showed that in normal individuals, only when serum E_2 levels were higher than 45 pg/ml, a strong positive correlation of E_2 with *hMLH1* gene expression was observed; and there was no significant correlation between *hMLH1* expression and serum E_2 level when E_2 levels were lower (E_2, < 45 pg/ml) (Jin *et al.*, 2010). In the current study of 18 CRC patients, only two patients' serum E_2 levels were higher than 45 pg/ml (a male, $E_2 = 69.16$ pg/ml; a femail, $E_2 = 70.18$ pg/ml). Therefore, the E_2 regulatory effects on MMR through ER-β and miRNA expression may be an important event that occurs early in tumorigenesis.

We observed that miR-135b expression was significantly higher in CRC tissues than in normal tissues, and miR-135b in exfoliated colonocytes

isolated from feces was significantly higher in CRC patients than in healthy volunteers (Koga *et al.*, 2010; Wu *et al.*, 2011). The first intron of the LEM domain containing 1 (*LEMD1*) gene contains miR-135b. Although the function of this gene is unclear, it has been reported that it exhibits high expression in CRC tissue compared to normal tissue (Yuki *et al.*, 2004). As miR-135b is located on 1q32.1 which frequently shows DNA copy number gain in CRC progression (Douglas *et al.*, 2004; Jones *et al.*, 2005), so the elevation of miR-135b level is common in CRC pathogenesis. The findings together may suggest that miR-135b could be a potential marker for CRC.

In summary, we found that E_2 through ER-β down-regulates miRNAs expression, especially miR-135b that may result in up-regulaion of MMR activity. Additional studies are warranted to further examine the effect of these changes in colorectal carcinogenesis.

Methods

Cell culture and transfection

COLO205, SW480 and MCF-7 cells were purchased from ATCC, and cultured as previously described (Jin *et al.*, 2010). COLO205 and SW480 cells were incubated in different concentrations of estradiol E_2 (Sigma-Aldrich., St Louis, MO) for 48 h; 100 nM of estrogen receptor antagonist ICI182,780 (Sigma-Aldrich., St Louis, MO) was then added followed by Annexin-V apoptosis assay. COLO205 cells were treated with 10 nM E_2 with a final volume of 0.01% ethanol for 0 h, 6 h, 12 h and 24 h to determine mRNA expression, or for 0 h, 24 h and 48 h to determine protein expression. SW480 and MCF-7 cells were treated with EtOH, 1 nM or 10 nM E_2, for 12 h alone or in combination with 100 nM ICI182,780 for 12 h. For the indicated experiments, cells were pretreated with 100 nM ICI182,780 for 6 h prior to EtOH or E_2 treatment. SW480 Cells were seeded in 6-well plates (Corning Costar Corp., Cambridge, MA) in a final volume of 3.0 ml of culture medium for 24 h before transfection. Cells grown to 70% confluence in tissue culture plates were transiently transfected with an empty pRST7 vector and pRST7-ER-β (Addgene., Cambridge, MA) using Lipofectamine (Invitrogen., Carlsbad, CA) as described by the manufacturer. The mammalian expression plasmid for ER-β (pRST7-ER-β) has been described previously (Hall and McDonnell, 1999). Western blotting was used to confirm the transfection efficiency after 24 h or 48 h of transfection. After 24 h of transfection, SW480 (*pERβ*) and SW480 (*vector*) were treated with EtOH or 10 nM E_2 for 12 h. After incubation, cells were harvested and used for quantitative reverse transcription-PCR (RT-qPCR) and Western blotting.

Subjects

A total of 18 patients - 9 males (age ranged from 31 to 73

yr, with a mean age of 53 yr) and 9 females (age ranged from 44 to 79 yr, with a mean age of 62 yr) diagnosed with CRC by colonoscopy were enrolled in this study from Beijing Military General Hospital. These patients were randomly selected from patient pool of the hospital's Gastrointestinal clinic; none of the subjects had undergone estrogen replacement therapy. Clinicopathologic characteristics of the patients are presented (Supplemental Data Table S1). Blood was taken half an hour after colonoscopy for determining the serum E_2 level. During colonoscopy, four or five samples from cancer tissues and adjacent non-cancer tissues, respectively, were obtained and stored in liquid nitrogen. The total RNA was isolated from frozen tissues with TRIzol (Invitrogen., Carlsbad, CA) according to the manufacture protocol. All tissue samples were reviewed and evaluated by a gastrointestinal pathological expert Sheng. In addition, the tissues used for RNA isolation were histologically identified to contain at least 70% of tumor tissue. The study was approved by ethics committee of the Beijing Military General Hospital. Informed consent was obtained from all study subjects.

Assessment of apoptosis by Annexin-V-FITC

E_2-induced apoptosis was measured with FITC-conjugated Annexin-V/PI assay kit (Abcam., Cambridge, MA) and flow cytometry. Cells were seeded in a 6-well plate, and following pretreatment, incubated in serum-free medium containing different concentrations of E_2 for 48 h. For estrogen receptor antagonist ICI182,780 inhibiting E_2-induced apoptosis, COLO205 cells were pretreated with 100 nM of ICI182,780 for 6 h prior to EtOH or E_2 treatment. 5×10^5 cells were briefly washed with ice cold phosphate buffer solution, resuspended in 100 μl of binding buffer, and stained with 5 μl of FITC-conjugated Annexin-V (10 mg/ml) and 10 μl of PI (50 mg/ml). The cells were incubated for 15 min at room temperature in the dark; 400 μl of binding buffer was then added followed by analysis with a FACScan flow cytometry (Becton--Dickinson).

Real-time PCR

The total RNA was extracted from cells of human cancer-surrounding tissue and cancer tissue with Trizol (Invitrogen., Carlsbad, CA). RNA concentrations were measured with a spectrophotometer (NanoDrop™ 2000) and RNA integrity was analyzed with gel electrophoresis. Before cDNA was synthesized with RevertAid™ First Strand cDNA Synthesis Kits (Fermentas., Vilnius, Lithuania), total RNA (one mg) was treated with DNase I (Invitrogen., Carlsbad, CA) to remove DNA contamination. The RT primers of miRNAs were as follows: miR-31RT, GTCGTATC-CAGTGCAGGGTCCGAGGTATTCGCACTGGATACGAC-AGCTAT; miR-155RT, GTCGTATCCAGTGCAGGGTCCG-AGGTATTCGCACTGGATACGACACCCCT; miR-135bRT, GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTG-GATACGACTCACAT; miR-203RT, GTCGTATCCAGTGCA-GGGTCCGAGGTATTCGCACTGGATACGACCTAGTG; miR-223RT, GTCGTATCCAGTGCAGGGTCCGAGGTATT-CGCACTGGATACGACTGGGGT.

Expression of mRNA and mature miRNAs was determined with the SYBR Green PCR Master Mix (TaKaRa.,

Dalian, China). miRNA expression was normalized according to U6-snRNA of the $2^{-\Delta\Delta CT}$-method (Pfaffl *et al*., 2001) and mRNA expression according to *β-Actin*. PCR was carried out in a volume of 20 μl, and PCR conditions were as following: denaturing at $95^{\circ}C$ for 30 s; denaturing at $95^{\circ}C$ for 5 s, reannealing at $60^{\circ}C$ for 34 s, elongation at $72^{\circ}C$ for 30 s, 40 cycles; finally elongation at $72^{\circ}C$ for 5 min. Primers used in this study were as follows: miR-31, sense, 5'-caAGGCAAGATGCTGGC-3'; miR-155, sense, 5'-cccTT-AATGCTAATCGTGA-3'; miR-135b, sense, 5'-gctTATGGC-TTTTCATTCCT-3'; miR-203, sense, 5'-ccaGTGAAATGTT-TAGGAC-3'; miR-223, sense, 5'-cccTGTCAGTTTGTCAA-AT-3'; miRNAs, anti-sense, 5'-GTGCAGGGTCCGAGGT-3'; U6, sense, 5'-GCTTCGGCAGCACATATACTAA-3'; anti-sense, 5'-AACGCTTCACGAATTTGCGT-3'; *hMLH1*, sense, 5'-TTCGTGGCAGGGGGTTATTCG-3'; antisense, 5'-GCCT-CCCTCTTTAACAATCACTT-3'; *hMSH2*, sense, 5'-GCTG-GAAATAAGGCATCCAAGG-3'; anti-sense, 5'-CACCAATG-GAAGCTGACATATCA-3'; *β-actin*, sense, 5'-CATGTACGT-TGCTATCCAGGC-3'; anti-sense, 5'-CTCCTTAATGTCAC-GCACGAT-3'.

Real-time quantification was performed on 5 microRNAs (miR-31, miR-135b, miR-155, miR-203 and miR-223) with an Applied Biosystems 7500 Sequence detection system. These 5 candidate miRNAs were selected because 1) they exhibited high expression in cancer tissue compared with normal tissue (Sarver *et al*., 2009; Wu *et al*., 2011), 2) their high expressions (miR-155, -223,-31) were significantly associated with MSI-H status in CRC patient (Earle *et al*., 2010), 3) they (miR-155) proved to regulate MMR genes (Valeri *et al*., 2010a), and 4) their expressions (miR-135b) in exfoliated colonocytes isolated from feces were significantly higher in CRC patients than in healthy volunteers (Koga *et al*., 2010). All RT-qPCRs were performed in triplicates. The differences in tissue samples between groups are presented with ΔCt or ΔΔCt (normal tissue as control), indicating the differences between the Ct values of the interested miRNA and the serum E_2 concentrations (Link *et al*., 2010).

Western blotting

Cells were washed with phosphate-buffered saline, and then lysed in extraction-buffer (Biosource, Camarillo, CA). Protein concentration was determined with BCA (Pierce, Rockford, IL). An aliquot of sample (25 mg) was separated on a 10% SDS-PAGE, and then transferred to a nitrocellulose membrane. The membrane was first incubated with the specific primary antibodies, ER-β, *hMLH1* and *hMSH2* (Proteintech Group., Chicago, IL) followed by incubation with horseradish-peroxidase linked by immunoglobulin G, and then visualized with chemiluminescence. The bands detected in Western blotting were analyzed with the Totallab 2.0 software.

Statistical analysis

Data analysis was performed with SPSS 11.5 software. The differences between two groups were analyzed with Student's *t* test, whereas the differences between multiple groups (more than two) were analyzed with ANOVA test. Correlation analyses between serum E_2 level and miRNA

expression, serum E_2 level and mRNA expression, and miRNA and mRNA expression were performed with Pearson's test, and logarithmic regression was used to calculate the R^2 and to create a regression equation. There is a strong correlation if the Pearson's P is < 0.5. R^2 value > 0.3 was regarded as the fitting model. Two-sided $P < 0.05$ was considered significant.

Supplemental data

Supplemental data include a table and can be found with this article online at http://e-emm.or.kr/article/article_files/SP-44-12-02.pdf.

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