O6-Methylguanine-DNA Methyltransferase Hypermethylation Modulated by 17β-Estradiol in Lung Cancer Cells

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Abstract. *Background: Our recent report indicated that MGMT hypermethylation is more common in squamous cell carcinomas (SCC) in males, and smokers than in adenocarcinomas (ADC) in females, and nonsmokers. More interestingly, MGMT hypermethylation in SCC and ADC was pronouncedly influenced by gender factor, not by smoking status. We questioned whether 17β-estradiol could modulate the machinery of promoter methylation to cause the gender difference of MGMT hypermethylation in lung cancer. Materials and Methods: Two MGMT hypermethylated Ch27 and H1355 lung cancer cell lines were treated with or without 17β-estradiol and the status of hypermethylation was examined by methylated specific methylation (MSP) as compared with both cells treated with demethylating agents, 5-AZA-dC (AZA) or TSA. Results: Our data showed that 17β-estradiol, similar to AZA, diminished the MGMT hypermethylation and restored MGMT mRNA expression, which was not observed in the case of TSA. Western blotting showed that 17β-estradiol markedly reduced DNMT1 expression in Ch27 and H1355 cells, but slightly reduced HDAC1 expression. Consequently, acetylated H3 and H4 histone levels were slightly increased by 17β-estradiol in both cell types. In addition, ChIP analysis revealed that 17β-estradiol simultaneously diminished the binding activity of both proteins on the MGMT promoter of both cell lines. Conclusion: 17β-Estradiol decreased DNMT1 and HDAC1 protein expressions and their binding activity on MGMT promoter, and this may partially contribute to the gender difference of MGMT hypermethylation in lung cancer.*

The silencing of *MGMT* is most likely attributed to hypermethylation of the MGMT promoter in several types of human carcinomas including lung cancer (1, 2). An *MGMT* transgenic mice model has demonstrated that K-ras mutation in lung tumors is significantly reduced in *MGMT* transgenic mice compared to that of non-transgenic mice, suggesting that *MGMT* inactivation by promoter methylation plays an important role in lung carcinogenesis (3). Our recent reports indicated that MGMT hypermethylation in lung cancer is more common in males, patients with squamous cell carcinomas (SCC), and smokers than in females, patients with adenocarcinomas (ADC), and nonsmokers. After stratification by gender, smoking status and tumor type, male nonsmokers in both tumor types had more prevalent *MGMT* hypermethylation than did female nonsmokers (53% *vs.* 31% for ADC, p=0.043; 65% *vs.* 29% for SCC, p=0.045), but the difference in *MGMT* hypermethylation was not observed between male smokers and nonsmokers in either tumor types (40% *vs.* 53% for ADC, p=0.326; 65% *vs.* 65% for SCC, p=0.990; (4)). More interestingly, *MGMT* hypermethylation may be associated with an increased occurrence of *p53* mutation including G:C to A:T transition and other types of *p53* mutations in lung cancer in males, but not in females. This result was consistent with our *p53* mutation database showing that lung cancer in nonsmoking males had higher *p53* mutation frequency than in corresponding female cases.

Our previous report indicated that a lower prevalence of estrogen receptor (*ER*) hypermethylation in lung cancer in females compared with that of males, might be due to increased acetylation of histone 3 (H3) and histone 4 (H4) of

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the *ER* promoter by 17β-estradiol (6). Thus, we speculated that *MGMT* hypermethylation in lung cancer in females could be modulated by 17β-estradiol much as *ER* hypermethylation was modulated by 17β-estradiol. To test this hypothesis, two lung cancer cell lines harboring with *MGMT* hypermethylation, Ch27 and H1355, were studied to see whether 17β-estradiol could modulate the machinery of promoter methylation to restore *MGMT* mRNA expression, such as the modulation of DNMT1 and HDAC1, which have been shown to be predominately involved in gene promoter methylation (7, 8). In addition, acetylated H3 and H4 levels and the binding activity of DNMT1 and HDAC1 on *MGMT* promoter were also evaluated by Western blotting and chromatin immuoprecipitation (ChIP), respectively.

Materials and Methods

Cell culture and reagents. Ch27 and H1355 cell lines (obtained from ATCC, USA) were maintained in RPMI-1640 containing 10% fetal bovine serum supplement with penicillin (100 units/ml) and streptomycin (100 μg/ml). The fetal bovine serum, penicillin, and streptomycin were purchased from HyClone Inc (HyClone, UT). Under estrogen-depleted conditions, cells were grown in an estrogen-depleted medium, consisting of phenol red-free RPMI-1640 from Sigma-Aldrich Inc. (Sigma-Aldrich, MO, USA), 10% charcoal-stripped fetal bovine serum, penicillin (100 units/ml), streptomycin (100 μg/ml), and 5% glucose as described previously. Cells were grown in a 37˚C humidified incubator with 5% CO_2. 5'-AZA-2'-deoxycytidine (AZA) and 17β-estradiol were purchased from Sigma-Aldrich, Inc. Trichostatin A (TSA) was purchased from Cayman Chemistry Inc. (MI USA). Antibodies against modified forms of acetyl-histone H3, acetyl-histone H4, and HDAC1 were purchased from Upstate Biotechnology, Inc. (NY USA), and antibodies specific for DNMT1 and cyclin D1 were purchased from IMGNEX (CA USA) and Santa Cruz Biotechnology Inc. (CA USA), respectively.

DNA extraction. Genomic DNA was isolated from cell lines by conventional phenol-chloroform extraction and ethanol precipitation, and then subjected to *p53* mutation and promoter methylation analysis as describes below.

Treatment with AZA or TSA. Cells were seeded at a density of 1×10^5/100-mm dish at 24 h prior to a treatment with 1 μM AZA or 10 nM 17β-estradiol or 10 nM TSA for up to 48 h. Fresh media containing drugs were added every 24 h. Treated or untreated cells from individual triplicate plates were harvested for analysis of their hypermethylation status using an MSP assay and *MGMT* mRNA and protein production analyzed by a real-time quantitative PCR and Western blot assay.

Methylation-specific PCR (MSP). The hypermethylation status of the *MGMT* promoter region was determined by a bisulfite modification (9) and a two-stage MSP assay (10). Primers used in stage I amplification of the *MGMT* gene were *MGMT*-forward (5'-GGATATGTTGGGATAGTT-3') and *MGMT*-Reverse (5'-CCA -AAAACCCCAAACCC-3'). The PCR amplification protocol for stage I was as follows: an initial reaction at 95˚C for 10 min, followed by 25 cycles of denaturation at 95˚C for 30 s, annealing at 52˚C for 30 s, and extension at 72˚C for 30 s, and then a final extension for 10 min. Primers used to selectively amplify unmethylated or methylated alleles of the *MGMT* genes in stage II PCR were: for unmethylated *MGMT*: forward primer, (5'-TTTGTGTTTTGATGTTTGTAGGTTTTTGT-3') and reverse primer, (5'-AACTCCACACTCTTCCAAAAACAAAACA-3'); for methylated *MGMT*: forward primer, (5'-TTTCGACGTTCGTAG GTTTTCGC-3') and reverse primer, (5'-GCACTCTTCCGAAAA CGAAACG-3'). In this round of PCR, the annealing temperature was 62˚C, and all of the cycling times were reduced to 15 s for 35 cycles. PCR products were run on 3% agarose gel and analyzed by ethidium bromide staining.

Preparation of RNA and real-time quantitative RT-PCR. The total RNA was isolated from Ch27 and H1355 lung cancer cells using 1 ml Trizol reagent (Invitrogen, CA, USA), followed by chloroform re-extraction and isopropanol precipitation. Three micrograms of total RNA from lung cancer cells were reverse transcribed using M-MLV reverse transcriptase (Promega, WI, USA) and oligo $d(T)_{15}$ primer. RTQ-PCR was performed in a final volume of 25 μl containing 1 μl of each cDNA template, 0.2 μM of each primer and 12.5 μl of a SYBR-Green master mix (Applied Biosystems, CA, USA). The primers were designed using the ABI Primer Express 3.0 Software (Applied Biosystems). The sequences of primers used were: *MGMT*, (5'-TGCACAGCCTGGCTGAATG-3') and (5'-GGTGAACGACTCTTGCTGGA-A-3'); *18S* gene (5'-TCGGAAC TGAGGCCAGA-3') and (5'-CCGGTCGGCATCG-TTTA-3'). Quantification was carried out using the comparative CT method and water was used as the negative control. An arbitrary threshold was chosen on the basis of the variability of the baseline. Threshold cycle (C_T) values were calculated by determining the point at which the fluorescence exceeded the threshold limit. C_T was reported as the cycle number at this point. The average of the target gene was normalized to *18S* rRNA as an endogenous housekeeping gene. After cycling, relative quantization of *MGMT* mRNA against an internal control, 18S, was conducted by the following ΔC_T method (11).

Western blotting. Cells were washed twice with PBS, on ice, before adding a protein lysis buffer (100 mM Tris, pH 8.0, 1% SDS). The protein concentration was determined by the Bradford assay (Bio-Rad, CA USA) using BSA as a standard. Total protein (20 μg) was loaded into each lane of the gel. After an electrophoretic transfer to a PVDF membrane, nonspecific binding sites were blocked with 5% nonfat milk in TBS-Tween 20. DNMT1, HDAC1, acetylated histone 3, acetylated histone 4, cyclin D1, and β-actin were detected by incubating the membrane with anti-DNMT1 (1:1,000), anti-HDAC1 (1:1,000), anti-acetylated histone 3 (1:1,000), anti-acetylated histone 4 (1:1,000), anti-cyclin D1 (1:1,000) and anti-β-actin (1:500,000) for 60 min at room temperature, followed by a subsequent incubation with a peroxidase-conjugated secondary antibody (1:5,000 dilution). Extensive washings with TBS-Tween 20 were performed between incubations to remove nonspecific binding. The protein bands were visualized using enhanced chemiluminescence (NEN Life Science, MA USA).

Chromatin immunoprecipitation (ChIP) assay. ChIP analysis was performed using a published procedure (12) with the following modifications. The immunoprecipitated DNA was ethanol precipitated and re-suspended in 25 μl water. Total input samples

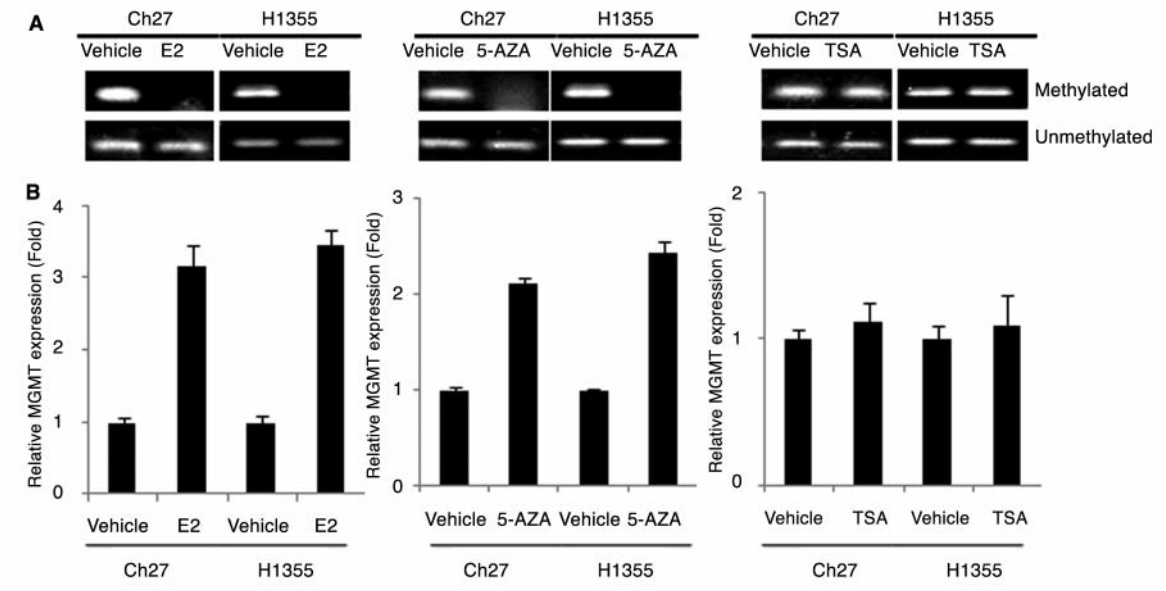

Figure 1. *Comparison of MGMT hypermethylation and its mRNA expression levels with or without 5-AZA-2'-dC, TSA, or E2 treatment. A, Cells were treated with 5-AZA-2'-dC (5-AZA, 1 μM), TSA (10 nM), or E2 (10 nM) for 48 h. MSP was used to assess the methylation status of the island with a primer specific for the unmethylated and methylated promoter in the MGMT CpG. B, Total RNA was isolated after the drug treatment and determined by real-time PCR. All experiments were carried out in duplicate. The values of the MGMT mRNA levels are indicated by relative mRNA fold-expression of MGMT. The copy number of MGMT cDNA was normalized to the copy number of 18S ribosome in each sample.*

were re-suspended in 100 μl water and diluted 1:100 before PCR analysis. PCR amplification of immunoprecipitated DNA was carried out with diluted aliquots, using the oligonucleotides (5'-GCCCCTAGAACGCTTTGC-3') and (5'-CAACACCTGG-GAG GCACTT-3') as primers, which encompass the 237 bp promoter region of *MGMT*. PCR products were run on 2% agarose gel and analyzed with ethidium bromide staining. All ChIP assays were duplicated.

Results

MGMT hypermethylation is modulated by 17β-estradiol to restore MGMT mRNA expression in lung cancer cells. Our previous report indicated that 17β-estradiol could modulate *ER* hypermethylation to restore *ER* mRNA expression in A549 lung cancer cells (6). In this study, we examined whether *MGMT* transcription silencing by promoter methylation could be reversed by 17β-estradiol. Two lung cancer cell lines with *MGMT* hypermethylation, Ch27 and H1355, were treated with 17β-estradiol (10 nM) for 48 h, and then MSP and real-time RT-PCR were performed to evaluate the status of *MGMT* hypermethylation and its mRNA expression levels. The effects of 17β-estradiol on *MGMT* hypermethylation and its mRNA expression were compared with those of the cells after treatment with demethylation agents, AZA or TSA. Our data showed that *MGMT* hypermethylation was abolished by 17β-estradiol and AZA in Ch27 and H1355 cells (Figure 1A). However, the demethylation effect of 17β-estradiol and AZA on *MGMT* hypermethylation was not observed under TSA treatment (Figure 1A). Meanwhile, *MGMT* mRNA expression in both cell lines was restored after treatment with 17β-estradiol and AZA (Figure 1B). *MGMT* mRNA levels of both cells after treatment with 17β-estradiol or AZA were elevated 2- to 3-fold compared with that of the solvent controls (Figure 1B), however no increase of *MGMT* mRNA expression was seen in either cell line after treatment with TSA (Figure 1B). These results clearly indicate that 17β-estradiol has a demethylation effect similar to AZA on *MGMT* promoter methylation, thus restoring *MGMT* transcription in Ch27 and H1355 lung cancer cells.

MGMT hypermethylation modulated by 17β-estradiol is mediated through reduced expression of DNMT1 and HDAC1 and their MGMT promoter-binding activity. DNMT1 and HDAC1 play a crucial role in gene silencing *via* reduced acetylation of H3 and H4 to silence gene transcription by promoter methylation (7, 8, 13-15). To elucidate whether the demethylation effect of 17β-estradiol on *MGMT* promoter methylation was mediated through the decrease of DNMT1 and HDAC1 expression, Western blotting analysis was performed. Our data showed that DNMT1 and HDAC1 decreased on 17β-estradiol treatment of Ch27 and H1355 cells (Figure 2). Interestingly, the effect of 17β-estradiol on DNMT1 expression was more pronounced than on that of

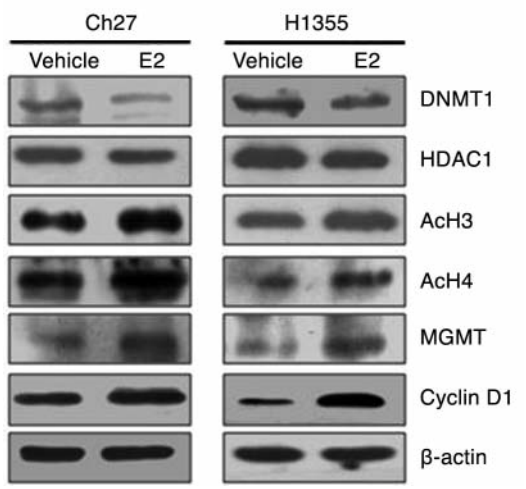

Figure 2. *Western blotting analysis of DNMT1, HDAC1, acetylated histone 3, acetylated histone 4 and MGMT expression levels in Ch27 and H1355 lung cancer cells after treatment with 10 nM E2 for 48 h. The expression of cyclin D1 was used as positive control for 17β-estradiol treatment.*

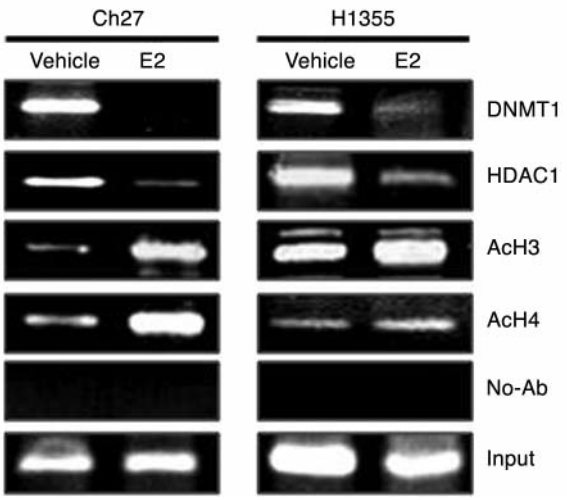

Figure 3. *ChIP assay for the binding activity of DNMT1, HDAC1, acetylated H3 (AcH3) and acetylated H4 (Ac H4) on the MGMT promoter. Chromatin was isolated and immunoprecipitated with an antibody specific for AcH3, AcH4, HDAC1 and DNMT1. Associated DNA was analyzed by PCR.*

HDAC1 (Figure 2). Meanwhile, 17β-estradiol slightly elevated acetylated H3 and H4 levels in both cell lines, consistent with the observation of the little effect of 17β-estradiol on HDAC1 expression (Figure 2). In this study, 17β-estradiol elevated cyclin D1 protein expression was used as a positive control (Figure 2).

To further verify whether 17β-estradiol could modulate the binding activity of DNMT1 and HDAC1 on the *MGMT* promoter, ChIP analysis was carried out. Our data showed that 17β-estradiol markedly suppressed the binding activity of DNMT1 and HADC1 on the *MGMT* promoter. Consequently, the binding activity of acetylated H3 and H4 on the *MGMT* promoter of Ch27 cells was also significantly elevated by 17β-estradiol, but increased acetylated H3 and H4 levels were not notably observed in H1355 cells (Figure 3). These results clearly indicate that *MGMT* hypermethylation, modulated by 17β-estradiol, might be mediated through the decrease of DNMT1 and HDAC1 expressions, consequently diminishing the binding activity of both proteins on the MGMT promoter.

Discussion

Our present study clearly shows that 17β-estradiol may reduce DNMT1 and HDAC1 protein expressions and the binding activity of both proteins on the *MGMT* promoter to restore *MGMT* transcription in lung cancer cells. Our previous reports showed that *MGMT* hypermethylation was more common in lung tumor tissues and lung cancer cells with *p53* mutation than in those without *p53* mutation. Based on our *p53* mutation database, no difference in *p53* mutation prevalence of lung cancer patients was observed between male smokers and male nonsmokers (87/263 (33.1%) *vs.* 25/61, (29.1%)), however, the prevalence of *p53* mutation in male nonsmokers was higher than in female nonsmokers (25/61 (29.1%) *vs.* 23/125 (18.4%)), respectively. We thus speculated that a higher 17β-estradiol concentration may play a role in the decrease of *MGMT* hypermethylation and p53 mutation in female patients. The concentration of 17β-estradiol (10 nM) used in the lung cancer cell experiments here was similar to those used in previous studies (16, 17). The 17β-estradiol concentration was higher than that of the physiological concentration in women, but 17β-estradiol concentration can increase significantly in women who have had hormone replacement therapy or use oral conceptive (18). Inconsistent results have been reported on the association of hormone replacement therapy with lung cancer risk (19-23). Interestingly, a recent study in Taiwan has clearly shown that women receiving hormone replacement therapy have a significantly lower risk for lung cancer (24). Even though the information for hormone replacement therapy and oral contraceptive in the study cases was not available, we could speculated that *MGMT* hypermethylation modulated by 17β-estradiol to decrease *p53* mutation occurrence might play a role in the decreased lung cancer risk in women, at least in Taiwanese women.

Histone acetylation plays an important role in remodeling chromatin structure to facilitate gene transcription. In this study and our previous study, it is possible that 17β-estradiol enhanced the overall histone acetylation to restore *MGMT* and *ER* mRNA expression in lung cancer cells (6). Sun *et al.*

(25) demonstrated that 17β-estradiol rapidly increased the level of acetylated histones by reducing the rate of histone deacetylation, whereas the rate of acetylation was not altered in breast cancer cells (25). In ps2 gene transcription regulated by the *ER* pathway, 17β-estradiol increased levels of acetylated H3 and H4 bound to the ps2 promoter and then the Sp1 bound to the promoter to up-regulate *ps2* gene transcription in *ER*-positive MCF-7 breast cancer cells (26). 17β-Estradiol was also involved in demethylation of CpG islands at the binding sites of an avian vitellogenin gene repressor (*MDBP-2*) to down-regulate the binding activity of MDBP-2 (27).

Our ChIP analysis clearly shows that 17β-estradiol markedly increased the acetylated H3 and H4 levels on the *MGMT* promoter, and that 17β-estradiol diminished the binding activity of HDAC1 and DNMT1 on the *MGMT* promoter (Figure 3B). A previous study showed that DNMT1 directly interacted with HDAC1, revealing that the process of DNA methylation mediated by DNMT1 may be dependent on or generate an altered chromatin state *via* histone deacetylase activity (7). In this study, DNMT1 expression was more pronounced than HDAC1, which was reduced by 17β-estradiol in lung cancer cells (Figure 2). The close connection between DNMT1 and HDAC1 may be relevant in promoting the modulation of the epigenetic state, just as *MGMT* hypermethylation is attenuated by 17β-estradiol.

The P1 region of the *p53* promoter contains a c-Myc/Max response element and 17β-estradiol has been shown to activate the transcription of *c-Myc* early in the G_1 phase (28, 29). Hurd *et al*. (30) indicated that the 17β-estradiol stimulus of c-Myc activates the P1 promoter of the *p53* gene to induce p53 protein expression in breast cancer cells (31). We also observed that 17β-estradiol treatment elevated protein expression of p53 and its downstream gene *p21* in Ch27 and H1355 lung cancer cells (data not shown). Phosphorylation of p53 has been shown to increase its sequence-specific DNA binding activity (31). More interestingly, 17β-estradiol treatment increased the levels of phosphorylated p53 at Ser15 in COS7 fibroblast cells (32). Therefore, DNMT1 protein expression, reduced by 17β-estradiol, may be partially mediated through increased *p53* transcription and phosphorylation. In this study, the modulation of DNMT1 by 17β-estradiol was more notable than that of HDAC1, and the failure of TSA in the restoration of *MGMT* mRNA expression reveals the possibility that *MGMT* promoter hypermethylation modulated by 17β-estradiol is predominately mediated through reduced DNMT1 expression.

In summary, *MGMT* hypermethylation modulated by 17β-estradiol may be mediated through reduced DNMT1 and HDAC1 expressions and the binding activity of both proteins on the *MGMT* promoter which then restores MGMT transcription in lung cancer cells. Therefore, we suggest that *MGMT* hypermethylation modulated by 17β-estradiol may partially account for the lower prevalence of *MGMT* hypermethylation and *p53* mutation in lung cancer of females compared with that of males, at least in Taiwanese lung cancer (4).

Acknowledgements

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