

## RESEARCH ARTICLE

# Oxidative Stress Induces Hypomethylation of LINE-1 and Hypermethylation of the RUNX3 Promoter in a Bladder Cancer Cell Line

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### Abstract

Increased oxidative stress and changes in DNA methylation are frequently detected in bladder cancer patients. We previously demonstrated a relationship between increased oxidative stress and hypomethylation of the transposable long-interspersed nuclear element-1 (LINE-1). Promoter hypermethylation of a tumor suppressor gene, runt-related transcription factor 3 (*RUNX3*), may also be associated with bladder cancer genesis. In this study, we investigated changes of DNA methylation in LINE-1 and *RUNX3* promoter in a bladder cancer cell (UM-UC-3) under oxidative stress conditions, stimulated by challenge with H<sub>2</sub>O<sub>2</sub> for 72 h. Cells were pretreated with an antioxidant, tocopheryl acetate for 1 h to attenuate oxidative stress. Methylation levels of LINE-1 and *RUNX3* promoter were measured by combined bisulfite restriction analysis PCR and methylation-specific PCR, respectively. Levels of LINE-1 methylation were significantly decreased in H<sub>2</sub>O<sub>2</sub>-treated cells, and reestablished after pretreated with tocopheryl acetate. Methylation of *RUNX3* promoter was significantly increased in cells exposed to H<sub>2</sub>O<sub>2</sub>. In tocopheryl acetate pretreated cells, it was markedly decreased. In conclusion, hypomethylation of LINE-1 and hypermethylation of *RUNX3* promoter in bladder cancer cell line was experimentally induced by reactive oxygen species (ROS). The present findings support the hypothesis that oxidative stress promotes urothelial cell carcinogenesis through modulation of DNA methylation. Our data also imply that mechanistic pathways of ROS-induced alteration of DNA methylation in a repetitive DNA element and a gene promoter might differ.

**Keywords:** Bladder cancer - DNA methylation - LINE-1 - *RUNX3* - oxidative stress

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### Introduction

Stepwise accumulations of genetic mutations and epigenetic alterations are vital events in the carcinogenic process. The main epigenetic change in cancer is the deregulation of DNA methylation, and both hypomethylated and hypermethylated DNA regions are observed throughout the cancer genome (Ehrlich and Lacey, 2013).

Bladder cancer, which is more prevalent in men, is the ninth most common cancer globally (Ploeg et al., 2009). Beside genetic mutations and epigenetic alterations, oxidative stress is also critically involved in the bladder cancer carcinogenesis. Increase in oxidative stress in patients with bladder cancer has been reported (Akçay et al., 2003, Opanuraks et al., 2010). Reactive oxygen species (ROS) directly damage the cellular DNA and promote tumor development not only through genetic mutations, but also through epigenetic alterations (Wachsmann, 1997). Global hypomethylation and regional (site-specific CpG island promoter) hypermethylation of

the tumor suppressor genes are demonstrated in bladder cancer (Sanchez-Carbayo, 2012). Methylation in repetitive DNA region reduces genomic instability by inactivation of transposons around the genome (Kazazian and Goodier, 2002), while methylation in the promoter decreases expression of the gene. Long interspersed nuclear element-1 (LINE-1), a tremendous transposon of human genome, has been considered as a good representative for measuring global methylation (Kazazian and Goodier, 2002). Hypomethylation of LINE-1 in bladder cancer has been demonstrated by various groups (Jurgens et al., 1996, Chalitchagorn et al., 2004, Wilhelm et al., 2010). Recently, we reported that patients with bladder cancer had increased oxidative stress and LINE-1 hypomethylation relative to the healthy controls (Patchsung et al., 2012). Furthermore, increased oxidative stress was found to correlate with decreased LINE-1 methylation both in patients and control subjects, suggested that hypomethylation of LINE-1 was in fact associated with increased oxidative stress (not cancer status per se). However, whether LINE-1 hypomethylation is a cause or effect of oxidative stress

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is remained to be elucidated. Although inflammation-induced hypomethylation of LINE-1 (by IL-6) was demonstrated (Gasche et al., 2011), oxidative stress-induced LINE-1 hypomethylation has not been explored.

Runt-related transcription factor 3 (*RUNX3*) is a tumor suppressor gene located at 1p36. *RUNX3* promoter is frequently hypermethylated in many cancers (Kim et al., 2004, Li et al., 2004, Lau et al., 2006). In bladder cancer, hypermethylation of *RUNX3* promoter in the tumor tissues was firstly reported by Kim et al. (2005). Later, they demonstrated that promoter hypermethylation of *RUNX3* was associated with the shorter survival suggested as a potential prognostic marker for bladder cancer (Kim et al., 2008). Wolff et al suggested that promoter hypermethylation of *RUNX3* could be a molecular clock of bladder tumor, as it increased with age, was not present in the normal urothelial cells and occurred early in the tumorigenesis (Wolff et al., 2008). Histone deacetylase inhibitor, nicotinamide was capable of re-expressing *RUNX3* gene and preventing bladder tumorigenesis in mouse (Kim et al., 2011). Recently, Yan and colleagues demonstrated that *RUNX3* methylation status could predict the risk of progression in patients with non-muscle-invasive bladder cancer (Yan et al., 2012). Moreover the *RUNX3* methylation status in histologically normal surrounding urothelium was associated with bladder tumor number and progression (Jeong et al., 2012). These studies underline the causal relationship between *RUNX3* promoter hypermethylation and bladder cancer development. Oxidative stress coincides with dysregulation of DNA methylation, however, the causal relationship between these two events is not well understood. Oxidative stress-induced *RUNX3* promoter hypermethylation in human colorectal cancer cell line was demonstrated (Kang et al., 2012), but induction of *RUNX3* promoter hypermethylation by ROS in bladder cancer cell line has not been investigated.

We hypothesize that oxidative stress may contribute to the carcinogenesis of bladder cancer via modification of DNA methylation in both genes and non-gene repetitive elements. We investigated herein changes of methylation patterns of LINE-1 and *RUNX3* promoter in bladder cancer cell line under the oxidative stress condition

## Materials and Methods

### Cell and culture conditions

Human bladder cancer cell line, UM-UC-3, was purchased from ATCC and cultured in Eagle's minimal essential medium (EMEM) (Gibco, CA, USA) supplemented with 10% fetal bovine serum (Gibco, CA, USA) under 37°C, 5% CO<sub>2</sub> and 95% humidity. The cells were challenged with H<sub>2</sub>O<sub>2</sub> (10 and 50 μM) for oxidative stress stimulation. Tocopheryl acetate (TA) was used as antioxidant for neutralizing oxidative stress. Before each experiment, cells were seeded in 24-well plate with density of 10,000 cells/cm<sup>2</sup> and cultured for 24 h. In each treatment condition, serum-free EMEM was used, and the conditioned medium was changed every 24 h. For TA treatment, the cells were pre-incubated with 50 μM or 300 μM of TA for 1 h prior to addition of H<sub>2</sub>O<sub>2</sub>.

### MTT assay and protein carbonyl determination

To assess the cytotoxicity of H<sub>2</sub>O<sub>2</sub>, MTT assay was carried out. UM-UC-3 cells were seeded in 96-well plate with 2x10<sup>5</sup> cells/well and cultured for 24 h. Cells were treated with varied concentrations of H<sub>2</sub>O<sub>2</sub> (10, 20, 50, 100 and 500 μM) for 72 h with change of conditioned media every 24 h. Solution of 0.1 M 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide or MTT was added and incubated with the cells for 2 h at 37°C. The cells were washed with PBS and lysed with dimethyl sulfoxide (100 μl/well). Absorbance of the formazan solution was measured at OD<sub>570</sub>. The obtained purple signal is proportionate to the amount of viable cells. The untreated cells were served as control and calculated as 100% viability.

The protein carbonyl content in whole cell lysate was used as an indicator of protein oxidation or oxidative protein damage. Cells in each condition were lysed with RIPA buffer (150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM EDTA, 50 mM Tris-Cl, pH 7.4). Procedure for protein carbonyl measurement was fully described in our previous report (Patchsung et al., 2012). The content of protein carbonyl in cell lysate was expressed per mg of total proteins (measured by the dye-binding method).

### DNA extraction and bisulfite treatment

DNA was extracted using the high-pure PCR template preparation kit (Roche, Basel, Switzerland) according to manufacturer's protocol. In brief, 40 ng/μl of extracted DNA was incubated with 2 M NaOH (5.5 μl) at 37°C for 10 min. After that, 10 mM hydroquinone, pH 5.0 (30 μl) (Sigma-Aldrich, MO, USA) and 3 M sodium bisulfite (520 μl) (Sigma-Aldrich, MO, USA) were added and incubated at 50°C for 16 h. Bisulfite-treated DNA was purified using Wizard DNA clean-up system (Promega, WI, USA). DNA was precipitated with absolute isopropanol (220 μl) containing 10 mM ammonium acetate (17 μl) and 20 mg/ml glycogen (1 μl) at -20°C for 2 h. DNA was harvested by centrifuge at 14,000 rpm (4°C) for 15 min. The DNA pellet was air dried and re-suspended with H<sub>2</sub>O (20 μl).

### Combined bisulfite restriction analysis (COBRA) PCR for LINE-1

LINE-1 methylation was determined using COBRA PCR assay as described earlier (Chalitchagorn et al., 2004, Patchsung et al., 2012). Firstly, the LINE-1 in bisulfite-treated DNA was amplified using the PCR reaction containing 2.5 mM MgCl<sub>2</sub>, 0.2 mM dNTP each, 1 U of Hotstart Taq DNA polymerase (Qiagen, Hilden, Germany) and 0.2 μM of each primer (F: 5'-CCG-TAA-GGG-GTT-AGG-GAG-TTT-TT-3' and R: 5'-RTA-AAA-CCC-TCC-RAA-CCA-AAT-ATA-AA-3'). PCR condition included 95°C 15 min, 35 cycles of 95°C denaturation, 50°C annealing, 72°C extension (1 min each) and final extension at 72°C for 7 min. The 160 bp LINE-1 amplicons (containing 2 CpG dinucleotides) were cut by 1 U of TaqI (TICGA) and TasI (IAATT), separated using 8% non-denaturing polyacrylamide gel electrophoresis, stained with SYBR Green and visualized by STORM scanner (GE healthcare Bio-sciences AB, Uppsala, Sweden). At

least quadruplet was done for each experiment.

As detected by COBRA, methylation status of the 2 CpG dinucleotides of LINE-1 loci was classified into four groups as follows: (i) LINE-1 loci containing 2 unmethylated CpGs ( ${}^u\text{C}_1{}^u\text{C}_2$ ); (ii) LINE-1 loci containing 2 methylated CpGs ( ${}^m\text{C}_1{}^m\text{C}_2$ ); (iii) LINE-1 loci containing 5'-methylated and 3'-unmethylated CpGs ( ${}^m\text{C}_1{}^u\text{C}_2$ ); and (4) LINE-1 loci containing 5'-unmethylated and 3'-methylated CpGs ( ${}^u\text{C}_1{}^m\text{C}_2$ ). The details for band intensity quantitation was fully described elsewhere (Patchsung et al., 2012). In brief, four bands that differed in their states of methylation, including 98 bp ( ${}^u\text{C}_1{}^u\text{C}_2$ ), 160 ( ${}^m\text{C}_1{}^m\text{C}_2$ ), 80 ( ${}^m\text{C}_2$ ) and 62 ( ${}^u\text{C}_1$ ) bp, were quantified (Figure. 1). The intensity of each band was divided by its paired length as followed: 160 bp/160 (A), 98 bp/94 (B), 80 bp/79 (C) and 62 bp/62 (D). The LINE-1 methylation level (total methylation) was calculated as the percentage of the methylated ( ${}^m\text{C}$ ) band intensity divided by the sum of the  ${}^m\text{C}$  and unmethylated ( ${}^u\text{C}$ ) band intensities,  $(\text{C}+\text{A}) / (\text{C}+\text{A}+\text{B}+\text{D}) \times 100$ . The percent of  ${}^u\text{C}_1{}^u\text{C}_2$  (hypomethylated loci) was calculated from the followed equation:  $\text{B} / ((\text{C}-\text{D}+\text{B})/2) + \text{A} + \text{D}) \times 100$ . The  ${}^m\text{C}_1{}^u\text{C}_2$  (partial methylated loci) was computed from the equation:  $\text{A} / ((\text{C}-\text{D}+\text{B})/2) + \text{A} + \text{D}) \times 100$ . The  ${}^m\text{C}_1{}^m\text{C}_2$  (hypermethylated loci) was computed from the equation:  $(\text{C}-\text{D}+\text{B})/2 / ((\text{C}-\text{D}+\text{B})/2) + \text{A} + \text{D}) \times 100$ .

#### Methylation-specific PCR (MSP) for RUNX3

RUNX3 methylation was measured by PCR using specific primers for methylated RUNX3 promoter (F: 5'-ATA-ATA-GCG-GTC-GTT-AGG-GCG-TCG-3' and R: 5'-GCT-TCT-ACT-TTC-CCG-CTT-CTC-GCG-3') and unmethylated RUNX3 promoter (F: 5'-ATA-ATA-GTC-GTT-GTT-AGG-GTG-TTC-3' and R: 5'-ACT-TCT-ACT-TTC-CCA-CTT-CTC-ACA-3') (Homma et al., 2006). The reaction contained 2.1 mM MgCl<sub>2</sub>, 0.2 mM dNTP, 4 μl of Q-solution, 1 U of Hotstart Taq DNA polymerase (Qiagen, Hilden, Germany) and 0.2 μM of each primer. The PCR reaction was started with 95°C 15 min followed by 35 cycles of 94°C denaturation for 30s, 59°C (for methylated primers) or 48°C (for unmethylated primers) annealing for 1 min, 72°C extension for 1 min. Final extension was performed at 72°C for 10 min. PCR products were separated in 8% non-denaturing polyacrylamide gel, stained with SYBR Green and visualized by STORM scanner.

#### Statistical analysis

Data presented as mean±SEM. One-way ANOVA and Bonferroni multiple comparison tests were used for testing the differences among groups. GraphPad Prism 5 and STATA version 10 were employed for graphs and statistical analyses. P value<0.05 was considered as statistically significant.

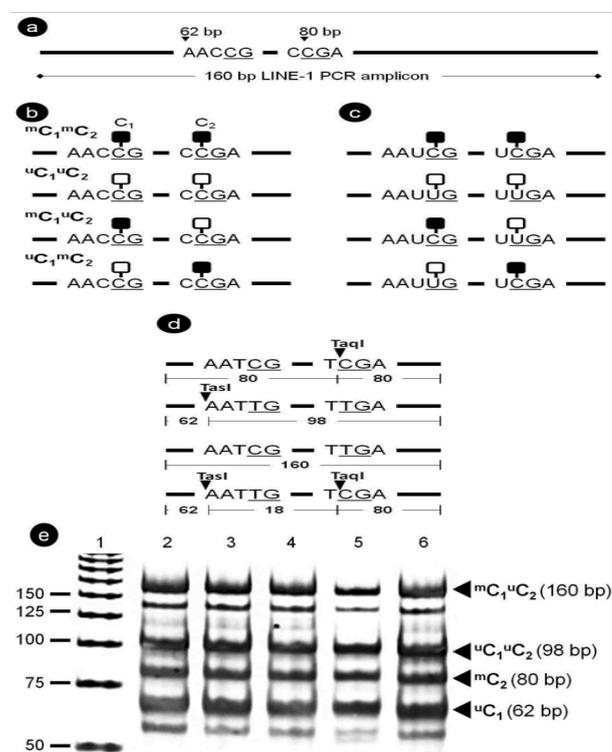
## Results

#### Cytotoxicity of hydrogen peroxide and induction of oxidative protein damage

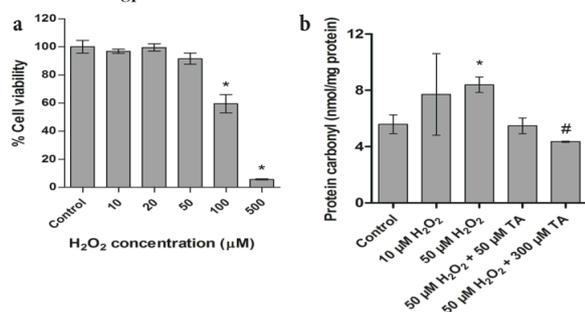
The concentration of H<sub>2</sub>O<sub>2</sub> for methylation experiments was chosen to ensure that the H<sub>2</sub>O<sub>2</sub> treatment was not

toxic to the UM-UC-3 cells. MTT assay was carried out to measure the viability of the cells treated with 10-500 μM of H<sub>2</sub>O<sub>2</sub> for 72 h. H<sub>2</sub>O<sub>2</sub> concentrations of 10, 20 and 50 μM did not significantly alter viability of the cells whereas higher concentrations caused significant decrease in cell viability (Figure 2a). H<sub>2</sub>O<sub>2</sub> concentrations of 100 μM and 500 μM significantly reduced cell viability to 58% and 5%, respectively (P<0.001 for both). Therefore, we decided to use 10 and 50 μM of H<sub>2</sub>O<sub>2</sub> for further investigation of DNA methylation.

Induction of oxidative stress in UM-UC-3 cells exposed to H<sub>2</sub>O<sub>2</sub> was investigated. Protein carbonyl content was measured as an indicator of oxidative protein damage. Level of protein carbonyl in H<sub>2</sub>O<sub>2</sub>-treated UM-UC-3 cells was significantly increased relative to untreated control cells (Figure 2b). Pretreatment with 300 μM TA was capable of preventing oxidative protein damage in H<sub>2</sub>O<sub>2</sub>-treated UM-UC-3 cells. This indicated that exposure of UM-UC-3 cells to H<sub>2</sub>O<sub>2</sub> caused increase in oxidative

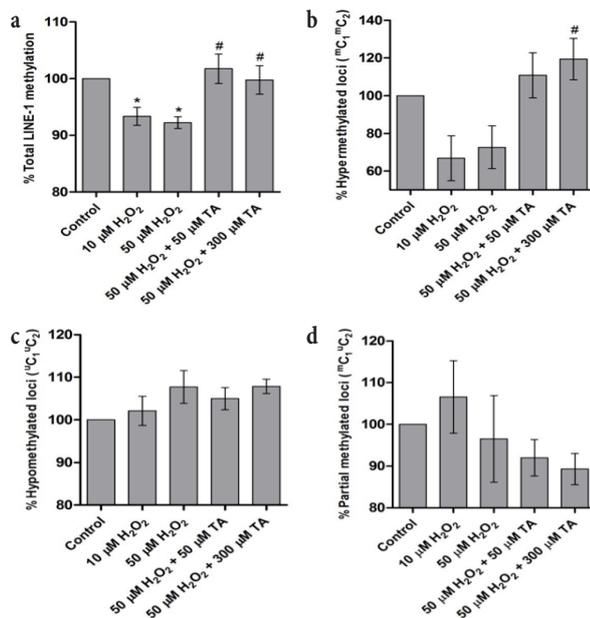


**Figure 1. Methylation Patterns of LINE-1 Detected by COBRA PCR.** (a) The detecting 5' UTR of the LINE-1 sequence contains two CpG dinucleotides (underline), and the PCR amplicon size of LINE-1 is 160 bp. (b) COBRA LINE-1 separated the detecting region into four products:  ${}^m\text{C}_1{}^m\text{C}_2$ ,  ${}^u\text{C}_1{}^u\text{C}_2$ ,  ${}^m\text{C}_1{}^u\text{C}_2$  and  ${}^u\text{C}_1{}^m\text{C}_2$ . The solid squares represent methylated C and the hollow squares represent unmethylated C. (c) After the bisulfite treatment, the unmethylated C residues are converted to U, but the methylated C residues remain unchanged. This leads to retention or loss of CpG containing restriction enzyme sites, respectively. (d) The PCR products are digested with TaqI (TICGA) and TasI (AATT) restriction enzymes. A TaqI positive digest yields two 80-bp DNA fragments, while a TasI positive digest yields a 62- and a 98-bp fragment. (e) Representative gel image for COBRA PCR LINE-1 assay. Lane 1: Base pairs marker, lane 2: untreated control cells, lane 3: 10 μM H<sub>2</sub>O<sub>2</sub>-treated cells, lane 4: 50 μM H<sub>2</sub>O<sub>2</sub>-treated cells, lane 5: 50 μM TA-pretreated/50 μM H<sub>2</sub>O<sub>2</sub>-treated cells, Lane 6: 300 μM TA-pretreated/50 μM H<sub>2</sub>O<sub>2</sub>-treated cells



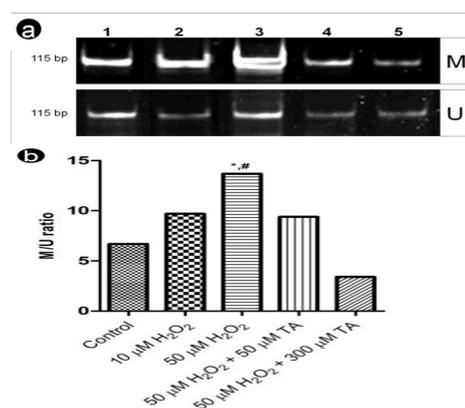
**Figure 2. Viability of UM-UC-3 Cells Exposed to Varied Concentrations of H<sub>2</sub>O<sub>2</sub> for 72 h and Oxidative Protein Damage in H<sub>2</sub>O<sub>2</sub>-treated UM-UC-3 Cells.**

At the concentrations of 10-50 μM, the cell viability did not significantly changed from the untreated control (a). In contrast, the viability was significantly reduced at concentrations of 100-500 μM (\*P<0.001 for both vs. control). Viability of the control condition was expressed as 100%. Level of protein carbonyl (indicator of oxidative protein damage) in H<sub>2</sub>O<sub>2</sub>-treated UM-UC-3 cells was significantly increased relative to untreated control cells (b). Pretreatment with TA was capable of preventing oxidative protein damage in H<sub>2</sub>O<sub>2</sub>-treated UM-UC-3 cells. This indicated that exposure of UM-UC-3 cells to H<sub>2</sub>O<sub>2</sub> caused increased in oxidative stress. Error bars indicate SEM. P<0.05: \* vs. control, #vs. 50 μM H<sub>2</sub>O<sub>2</sub>



**Figure 3. LINE-1 Methylation Status in H<sub>2</sub>O<sub>2</sub> and/or TA-treated UM-UC-3 Cells.**

(a) The total LINE-1 methylation (%) in H<sub>2</sub>O<sub>2</sub>-treated cells was significantly decreased compared with the untreated control cells (P=0.011 for 10 μM, P=0.002 for 50 μM). The total LINE-1 methylation level was significantly restored after pre-treated with TA compared to cells treated with 50 μM H<sub>2</sub>O<sub>2</sub> (P=0.002 and P=0.022 for 50 μM and 300 μM TA, respectively). (b) The percent of mC<sub>1</sub>mC<sub>2</sub> loci tended to decrease in H<sub>2</sub>O<sub>2</sub>-treated cells and increase in TA-pretreated/H<sub>2</sub>O<sub>2</sub>-treated cells, although statistical significance were not revealed. However, the % mC<sub>1</sub>mC<sub>2</sub> in 300 μM TA-pretreated/50 μM H<sub>2</sub>O<sub>2</sub>-treated cells significantly increased compared to the 10 μM H<sub>2</sub>O<sub>2</sub>-treated cells (P=0.028). (c) The % uC<sub>1</sub>uC<sub>2</sub> tended to increase in H<sub>2</sub>O<sub>2</sub>-treated cells and decrease in the TA-pretreated/H<sub>2</sub>O<sub>2</sub>-treated cells (P>0.05). (d) Likewise, the % mC<sub>1</sub>uC<sub>2</sub> tended to increase in H<sub>2</sub>O<sub>2</sub>-treated cells and decrease in the TA-pretreated/H<sub>2</sub>O<sub>2</sub>-treated cells (P>0.05). Control untreated cells were expressed as 100%. Error bars indicate SEM. P<0.05: \*vs. control, #vs. 50 μM H<sub>2</sub>O<sub>2</sub>



**Figure 4. RUNX3 Promoter Methylation Status of H<sub>2</sub>O<sub>2</sub> and/or TA-treated Cells.**

(a) Representative gel of PCR products of methylated RUNX3 (M) and unmethylated RUNX3 (U) is shown. (b) The ratio of methylated RUNX3/unmethylated RUNX3 (M/U ratio) for control (lane 1), 10 μM H<sub>2</sub>O<sub>2</sub> (lane 2), 50 μM H<sub>2</sub>O<sub>2</sub> (lane 3), 50 μM H<sub>2</sub>O<sub>2</sub>+50 μM TA (lane 4) and 50 μM H<sub>2</sub>O<sub>2</sub>+300 μM TA (lane 5) were of 6.7, 9.7, 13.7, 9.4 and 3.4 respectively. The methylation/unmethylation ratio was significantly increased in 50 μM H<sub>2</sub>O<sub>2</sub>-treated condition compared with the untreated control. Significant reduction of methylated/unmethylated RUNX3 ratio after pretreatment with 300 μM TA was observed. P<0.05: \*vs. untreated control, #vs. 50 μM H<sub>2</sub>O<sub>2</sub>+300 μM TA

stress.

*H<sub>2</sub>O<sub>2</sub> induces LINE-1 hypomethylation*

Change of LINE-1 methylation level in H<sub>2</sub>O<sub>2</sub>-treated UM-UC-3 cells was explored. The percent of total methylation of LINE-1 in 10 and 50 μM H<sub>2</sub>O<sub>2</sub>-treated cells was significantly decreased to 93.35% (P=0.011) and 92.23% (P=0.002), respectively, compared with the untreated condition (100%) (Figure 3a). After pretreatment with 50 and 300 μM TA, the LINE-1 methylation level was significantly restored to 101.76% (P=0.002 vs. 50 μM H<sub>2</sub>O<sub>2</sub>) and 99.78% (P=0.022 vs. 50 μM H<sub>2</sub>O<sub>2</sub>), respectively. These results indicated that hypomethylation of LINE-1 was induced by ROS, and introduction of the antioxidant was capable of preventing this effect.

The percent of hypermethylated loci (mC<sub>1</sub>mC<sub>2</sub>) tended to decrease in H<sub>2</sub>O<sub>2</sub>-treated cells and increase in TA-pretreated/H<sub>2</sub>O<sub>2</sub>-treated cells, although statistical significance were not observed. However, the % mC<sub>1</sub>mC<sub>2</sub> in 300 μM TA-pretreated/50 μM H<sub>2</sub>O<sub>2</sub>-treated cells was significantly increased compared to the 10 μM H<sub>2</sub>O<sub>2</sub>-treated cells (P=0.028) (Figure 3b). The percent of hypomethylated loci (uC<sub>1</sub>uC<sub>2</sub>) tended to increase in H<sub>2</sub>O<sub>2</sub>-treated cells and decrease in the TA-pretreated/H<sub>2</sub>O<sub>2</sub>-treated cells, although there was no statistical significance (Figure 3c). Likewise, the percent of partially methylated loci (mC<sub>1</sub>uC<sub>2</sub>) tended to increase in H<sub>2</sub>O<sub>2</sub>-treated cells and decrease in the TA-pretreated/H<sub>2</sub>O<sub>2</sub>-treated cells, although they were not significantly different (Figure 3d).

*H<sub>2</sub>O<sub>2</sub> induces RUNX3 promoter hypermethylation*

UM-UC-3 cells treated with 10 and 50 μM H<sub>2</sub>O<sub>2</sub> for 72 h showed increase in methylation level of RUNX3 promoter (Figure 4a). The ratio between methylated RUNX3/unmethylated RUNX3 was increased from 6.7

(control) to 9.7 (10  $\mu\text{M}$   $\text{H}_2\text{O}_2$ ) and 13.7 (50  $\mu\text{M}$   $\text{H}_2\text{O}_2$ ) (Figure 4b). The methylation/unmethylation (M/U) ratio was significantly increased in 50  $\mu\text{M}$   $\text{H}_2\text{O}_2$ -treated condition compared to the untreated control. On the contrary, pretreatment with 50  $\mu\text{M}$  and 300  $\mu\text{M}$  TA caused decrease in M/U ratio from 13.7 to 9.4 and 3.4, respectively. Significant reduction of M/U ratio after pretreatment with 300  $\mu\text{M}$  TA was revealed (Figure 4b).

## Discussion

Increase in ROS production and alterations of DNA methylation are well recognized to vitally involve in the carcinogenic process. However, the causal link between these two events is unknown. We investigated the effect of  $\text{H}_2\text{O}_2$ , a representative of ROS, on methylation changes of LINE-1 and *RUNX3* promoter in bladder cancer UM-UC-3 cell line. Dose of  $\text{H}_2\text{O}_2$  lower than 50  $\mu\text{M}$  was proved to be non-toxic to the UM-UC-3 cells. Thus, changes of methylation levels observed in the present experiments were not caused by cell death, but the degree of oxidative stress. We decided to use 10 and 50  $\mu\text{M}$   $\text{H}_2\text{O}_2$  for investigating the DNA methylation change. Increase in protein carbonyl content in  $\text{H}_2\text{O}_2$ -treated cells was demonstrated to ensure an induction of oxidative stress. Many studies reported  $\text{H}_2\text{O}_2$  concentrations in the urine, ranged between 1 and 25  $\mu\text{M}$  in normal volunteers (except for a few circumstances that up to 100  $\mu\text{M}$  was reported) (Halliwell et al., 2004). Therefore, doses of  $\text{H}_2\text{O}_2$  used in our experiments were not extremely higher than the physiological dose, and these doses were capable of inducing cellular oxidative stress.

We demonstrated for the first time that LINE-1 was hypomethylated in bladder cancer cells exposed to  $\text{H}_2\text{O}_2$ , although Gashe et al had demonstrated that the LINE-1 hypomethylation was induced by IL-6 in oral cancer cells (Gasche et al., 2011). The current result warranted that oxidative stress experimentally caused LINE-1 hypomethylation. It might, at least in part, explain our previous clinical finding that showed that bladder cancer patients who had enhanced oxidative stress coincidentally had LINE-1 hypomethylation (Patchsung et al., 2012). In addition to  $\text{H}_2\text{O}_2$ , we had tried to experimentally induce LINE-1 hypomethylation in bladder cancer cell by inflammatory cytokine (TNF- $\alpha$ ) similar to the study by Gashe et al. (2011). Unfortunately, we found no change of LINE-1 methylation in UM-UC-3 cells exposed to TNF- $\alpha$  (data not shown). It is known that hypomethylation of LINE-1 causes overexpression of LINE-1 transposons leading to induction of genomic instability and eventually malignancy transformation (Kazazian and Goodier, 2002). We showed that LINE-1 hypomethylation was capable of counteracting by an antioxidant, tocopheryl acetate. This data suggest that hypomethylation of LINE-1 by ROS is a reversible and preventable process. Antioxidant might have an additional anti-carcinogenic effect through prevention of LINE-1 hypomethylation.

Change of patterns of LINE-1 methylation under oxidative stress was also investigated. We found that the  $^m\text{C}_1^m\text{C}_2$  loci were decreased, while  $^m\text{C}_1^u\text{C}_2$  and  $^u\text{C}_1^u\text{C}_2$  loci were increased in  $\text{H}_2\text{O}_2$ -treated cells. Reversely, increased

in  $^m\text{C}_1^m\text{C}_2$  loci, decrease in  $^m\text{C}_1^u\text{C}_2$  and slight decrease in  $^u\text{C}_1^u\text{C}_2$  loci were found in TA pretreatment condition. Based on the current findings we hypothesize that the decreased LINE-1 methylation in  $\text{H}_2\text{O}_2$ -treated cells may be due to stepwise demethylation of  $^m\text{C}_1^m\text{C}_2$  to form  $^m\text{C}_1^u\text{C}_2$  and subsequently to  $^u\text{C}_1^u\text{C}_2$ . Further kinetic experiment is required to warrant this.

Although it is not precisely known, several mechanisms of how oxidative stress reduces DNA methylation have been proposed. DNA methyltransferase (DNMT) requires S-adenosylmethionine (SAM) as a methyl donor for methylation reaction, and SAM is synthesized from methionine. In oxidative stress, homocysteine is used for synthesizing glutathione (GSH) to scavenge ROS, and the synthesis of homocysteine requires SAM. Increase in GSH synthesis under oxidative stress condition, therefore, leads to depletion of SAM to be used in the DNA methylation reaction (Hitchler and Domann, 2007). Reduction of GSH that causes reduction of SAM and subsequently DNA methylation is demonstrated (Lertratanangkoon et al., 1996). Another mechanism of ROS-induced loss of DNA methylation involves DNA repairing process. An increase in NAD<sup>+</sup>/NADH ratio in oxidative environment up-regulates the ten eleven translocation (TET), a hydroxylase enzyme that hydroxylates the 5-methylcytosine to form the 5-hydroxymethylcytosine (Chia et al., 2011). The 5-hydroxymethylcytosine is subsequently fixed by the base excision repair to substitute the 5-hydroxymethylcytosine with a new unmethylated cytosine, hence resulting in reduction of DNA methylation (Cedar and Bergman, 2012). In addition, ROS oxidizes the guanine base to yield 8-hydroxyguanine or 8-oxoguanine. This oxidative lesion in CpG dinucleotides strongly inhibits the methylation activity of DNMT (Weitzman et al., 1994). In sum, these three mechanisms are theoretically possible, and in cancer cells all of these pathways may act in concert to cause DNA hypomethylation. The precise mechanisms of ROS-induced LINE-1 hypomethylation in bladder cancer remain to be elucidated.

*RUNX3* is a tumor suppressor gene that is strongly associated with bladder cancer carcinogenesis and tumor progression (Kim et al., 2005; 2008, Jeong et al., 2012; Yan et al., 2012). We found that ROS caused increase in methylation of *RUNX3* promoter, and this hypermethylation was prevented by tocopheryl acetate. Kang et al found that *RUNX3* promoter in colon cancer cells was hypermethylated under the oxidative stress condition, possibly due to increases in DNMT and histone deacetylase activity (Kang et al., 2012). O'Hagan et al. (2011) proposed that increase in NAD<sup>+</sup>/NADH ratio in oxidative condition induces the formation of a large silencing complexes containing DNMTs and polycomb repressive complex 4 at CpG-rich promoter (O'Hagan et al., 2011), and the high activity of DNMTs in this polycomb complex cause hypermethylated promoter. However, ROS-induced *RUNX3* promoter hypermethylation requires further experimental proof.

Limitations of the present study should be mentioned. We did not have the transcript expression data of the hypomethylated LINE-1 and hypermethylated *RUNX3*. Expressions of DNMTs and histone deacetylase did not

investigate. Additionally, only one cell line was tested. Further study should be accomplished to warrant our present novel findings.

In conclusion, we firstly demonstrated that bladder cancer cells manifested LINE-1 hypomethylation and *RUNX3* promoter hypermethylation under the oxidative stress condition. The present findings support the hypothesis that oxidative stress promotes carcinogenesis through global DNA hypomethylation and promoter hypermethylation of tumor suppressor genes. Attenuation of cellular oxidative stress by antioxidants may be beneficial for preventing alterations of DNA methylation and hence malignant transformation. Our current data also implied that mechanistic pathways of ROS-induced alteration of DNA methylation in gene promoters and repetitive DNA elements might be different.

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