RESEARCH ARTICLE

Increased Oxidative Stress and *RUNX3* Hypermethylation in Patients with Hepatitis B Virus-Associated Hepatocellular Carcinoma (HCC) and Induction of *RUNX3* Hypermethylation by Reactive Oxygen Species in HCC Cells

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Abstract

Promoter hypermethylation of the runt-related transcription factor 3 (*RUNX3*) gene is associated with increased risk for hepatocellular carcinoma (HCC). Oxidative stress plays a vital role in both carcinogenesis and progression of HCC. However, whether oxidative stress and *RUNX3* hypermethylation in HCC have a cause-and-effect relationship is not known. In this study, plasma protein carbonyl and total antioxidant capacity (TAC) in patients with hepatitis B virus (HBV)-associated HCC (n=60) and age-matched healthy subjects (n=80) was determined. *RUNX3* methylation in peripheral blood mononuclear cells (PBMC) of subjects was measured by methylation-specific PCR. Effect of reactive oxygen species (ROS) on induction of *RUNX3* hypermethylation in HCC cells was investigated. Plasma protein carbonyl content was significantly higher, whereas plasma TAC was significantly lower, in HCC patients than healthy controls. Based on logistic regression, increased plasma protein carbonyl and decreased plasma TAC were independently associated with increased risk for HCC. PBMC *RUNX3* methylation in the patient group was significantly greater than in the healthy group. *RUNX3* methylation in hydrogen peroxide (H_2O_2)-treated HepG2 cells was significantly higher than in untreated control cells. In conclusion, increase in oxidative stress in Thai patients with HBV-associated HCC was demonstrated. This oxidative increment was independently associated with an increased risk for HCC development. *RUNX3* in PBMC was found to be hypermethylated in the HCC patients. *In vitro*, *RUNX3* hypermethylation was experimentally induced by H_2O_2. Our findings suggest that oxidative stress is a cause of *RUNX3* promoter hypermethylation in HCC cells.

Keywords: HCC, HBV, oxidative stress, ROS, *RUNX3* hypermethylation, DNA methylation

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Introduction

Liver cancer is a leading cancer in Thailand comprising of two main forms. One is cholangiocarcinoma, and the other is hepatocellular carcinoma (HCC). HCC in Thailand is principally associated with chronic hepatitis B virus (HBV) infection (Tangkijvanich et al., 1999), however molecular mechanism of HBV-associated HCC development in Thai patients is not fully understood. Beside chronic inflammatory activation, oxidative stress is considerably increased in HBV-associated HCC patients (Tsai et al., 2009, Nair et al., 2010, Zhao et al., 2011), and has been believed to play an important role in the development of viral-induced HCC (Marra et al., 2011, Higgs et al., 2014). In addition, oxidative stress is found to increase during the replication of HBV in cell culture model (Severi et al., 2006).

Oxidative stress, a condition with overwhelming generation of reactive oxygen species (ROS) and/or inadequacy of antioxidants, exerts a tumorigenic role to promote both genetic mutation and epigenetic alteration (Franco et al., 2008, Ziech et al., 2011). An epigenetic hallmark that is found in all carcinomas including HCC is an alteration of DNA methylation (Herceg and Paliwal, 2011). There are two types of DNA methylation alterations in cancers viz. genome-wide or global hypomethylation and promoter hypermethylation of tumor suppressor genes (TSG). Silencing of TSG via DNA methylation is well recognized in the carcinogenesis of HCC (Sceusi et al., 2011). Runt-related transcription factor 3 (*RUNX3*) is one of TSG that vitally involves in the HCC carcinogenesis and progression. Promoter hypermetnylation of *RUNX3* is associated with increased risk for HCC (Yang et al., 2014, Zhang et al., 2015). *RUNX3* expression is decreased

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in HCC tissues as well as HCC cell lines (Li and Jiang, 2011). Loss of *RUNX3* expression is associated with the progression of tumor, as it is able to induce epithelial-mesenchymal transition (EMT) in the low-EMT HCC cells (Tanaka et al., 2012). Up to now, cause and regulation of *RUNX3* hypermethylation in HCC is not known.

Oxidative stress-induced aberrations of DNA methylation in HCC has been hypothesized and demonstrated (Nishida and Kudo, 2013). ROS-induced hypermethylation of E-cadherin promoter is demonstrated in HCC cell lines (Lim et al., 2008). We previously demonstrated an induction of *RUNX3* hypermethylation by ROS in bladder cancer cells (Wongpaiboonwattana et al., 2013). Hitherto, causative relationship between ROS and *RUNX3* hypermethylation in HCC, especially HBV-associated HCC, has not been investigated.

In the present study, oxidative stress and *RUNX3* hypermethylation in HBV-associated HCC patients were investigated. Experimentally, whether ROS was able to induce *RUNX3* hypermethylation in HCC cells was investigated.

Materials and Methods

Patients and specimen collection

A total of 140 subjects divided into HCC (n=60) and healthy (n=80) groups were recruited for the study. Means age between these two groups (52.33±7.91 *vs*. 50.59±5.54 years old) were not significantly different (Table 1). There were 52 (86.67%) men and 8 (13.33%) women in the HCC groups. The healthy control group was consisted of 50 (62.5%) men and 30 (37.5%) women (P=0.001 *vs*. HCC group). All HCC patients were serologically proof (including DNA test) to have chronic HBV infection, considered as HBV-associated HCC. HBsAg was positive, but anti-hepatitis C virus was negative in all cases. Of 60 patients, 52 had data of cirrhosis and staging. Most of patients (78.85%) had cirrhotic liver. According to Barcelona-Clinic Liver Cancer (BCLC) staging system, patients in stage 0, A, B and C were accounted for 2 (3.85%), 7 (13.46%), 19 (36.54%) and 24 (46.15%), respectively (Table 1).

Blood samples were preoperatively collected, representing as pre-treatment samples. Plasma was separated, and DNA was isolated from peripheral blood mononuclear cells (PBMC). Healthy subjects were blood donors at Thai Red Cross, Bangkok, Thailand. Blood samples leftover from the routine blood test were used for plasma and DNA isolations. Informed consents were received from all participants prior to collection of specimen. Research protocol was approved by the Ethics Committee, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand.

Protein carbonyl determination

Protein carbonyl content, an indicator of oxidative protein damage (protein oxidation), was measured in plasma samples and cell lysate. The procedure for protein carbonyl measurement in plasma was fully described in our previous report (Patchsung et al., 2012). For cell lysate, cells were lyzed with RIPA buffer, and the lysate was centrifuged at 4,500 xg for 10 min to collect supernatant. Protein concentration was determined by Bradford assay (Bio-Rad Laboratories, CA, USA). Protein carbonyl in cell lysate was measured in a similar way to the procedure performed in plasma samples. Experiments were performed at least in triplicate.

Total antioxidant capacity (TAC) measurement

TAC was measured using 2,2'-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid (ABTS) method (Floegel et al., 2011). For quality control, absorbance of ABTS radical solution at 734 nm between lots was adjusted to 0.650±0.020. Plasma or cell lysate samples were diluted (1:5) in distilled water. Samples or water (blank) (20 μL) were added to ABTS solution (980 μL). The mixture was mixed well and incubated at 37oC for 10 min. Absorption (A) at 734 nm was measured. Percentage of antioxidant activity (%AA) of sample was calculated from: $\%AA=((A_{blank} - A_{sample})/A_{blank}) \times 100$. Vitamin C standard with known concentrations (0, 0.25, 0.5 and 1 mM) were used for creating a standard curve (%AA *vs*. vitamin C concentration) (R^2=0.9253). TAC of each sample was derived from standard curve and expressed as vitamin C equivalent antioxidant capacity (VCEAC) (mg/L). Each sample was measured in duplicate.

Cell culture condition

HepG2 cells gifted from Dr. Sunchai Payungporn were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (Gibco, CA, USA), 1% Pen-Strep under 37°C, 5% CO_2, and 95% humidity. H_2O_2 (Merck Schuchardt OHG, Hohenbrunn, Germany) was used to stimulate cellular oxidative stress. N-acetylcysteine (NAC) (Calbiochem, San Diego, CA, USA) at 50 μM was used as antioxidant to attenuate oxidative stress in the H_2O_2-treated cells.

Cell viability assay

HepG2 cells were plated in a 96-well plate ($2x10^5$ cells/well). Confluent cells were treated with varied concentrations of H_2O_2 (0, 10, 20, 30, 40, 50, 70, 100, and 200 μM) in serum-free medium for 72 h. After washing, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (0.5 mg/mL) was added and incubated for 4 h at 37°C, 5% CO_2, and 95% humidity. MTT solution was discarded and dimethyl sulfoxide (100 μL/well) was added to solubilize the purple formazan crystals. Absorption at 570 nm was measured. Untreated cells were used as controls and expressed as 100% viability.

DNA extraction and bisulfite treatment

PBMC was isolated from blood samples. DNA was extracted using genomic DNA extraction kit (RBC Bioscience, New Taipei City, Taiwan) according to the manufacturer's procedure. DNA concentration was measured using spectrophotometer (NanoDrop 2000c, Wilmington, DE, USA). Bisulfite conversion of DNA (250 ng) was performed using EZ DNA Methylation-Gold™ kit (Zymo Research, Irvine, CA, USA). The bisulfite-treated DNA was collected and kept at -20°C for further analysis.

Measurement of RUNX3 methylation by methylation-specific PCR (MSP)

RUNX3 methylation was measured by MSP method (Homma et al., 2006, Zhang et al., 2009). Methylated primers were F: 5'-ATA ATA GCG GTC GTT AGG GCG TCG-3' and R: 5'-GCT TCT ACT TTC CCG CTT CTC GCG-3' (115 bp). Unmethylated primers were F: 5'-ATA ATA GTC GTT GTT AGG GTG TTC-3' and R: 5'-ACT TCT ACT TTC CCA CTT CTC ACA-3' (124 bp). The PCR reaction contained $MgCl_2$ (6.7 mM for methylated primers, 5.0 mM for unmethylated primers), 1.0 mM dNTP, 10 mM β-mercaptoethanol, 0.1 μM primers and 1 U of i-TaqTM plus DNA polymerase (iNtRON Biotechnology, Korea). Initial denaturation was carried out at 95°C for 15 min using PCR thermal cycler (Eppendorf Mastercycler® Pro S, Germany). PCR of 35 cycles was performed as followed: denaturation at 94°C for 30 sec, annealing at 57°C for 1 min and extension at 72°C for 1 min. Final extension was performed at 72°C for 10 min. PCR products were separated in 3% agarose gel (100 volts, 70 min), stained with ethidium bromide and visualized using Molecular Imager Gel DocTM XR+ (Bio-Rad Laboratories). Band intensity was measured by Image Lab™ software (Bio-Rad Laboratories). Level of *RUNX3* methylation was expressed as methylation-to-unmethylation (M/U) band intensity ratio.

Statistical analysis

Data are presented as mean±standard deviation (SD) or median (interquatile range, IQR) as appropriate. Two-sample t-test or Mann-Whitney test was used to test the difference between two independent groups. One-way ANOVA followed by Tukey multiple comparison test or Kruskal-Wallis followed by Dunns test was used for testing the differences among three or more groups. Logistic regression was performed to obtain odds ratio (OR) adjusted for age and sex. Stata version 10 (College Station, TX) and GraphPad Prism 5 softwares (GraphPad, La Jolla, CA) was employed for graphs and statistical analyses. P value <0.05 was considered statistically significant.

Results

Increased oxidative stress in HBV-associated HCC patients

Protein carbonyl and TAC were used as biomarkers for oxidative stress. Plasma protein carbonyl content in HBV-associated patients was significantly elevated, as compared to the age-matched healthy controls (Figure 1). In contrast, plasma TAC in HCC patients was significantly decreased, as compared to the healthy controls.

Because control group had more females than HCC group, we performed logistic regression to control confounding factors and quantify the strength of association of oxidative stress biomarkers with HCC. The β-coefficient of plasma protein carbonyl and TAC controlled for age and sex were 1.64 (95%CI: 0.55 - 2.73, P=0.003) and -0.02 (95%CI:-0.02 - -0.01, P<0.001), and their adjusted OR were 5.15 (95%CI: 1.73 - 15.39) and 0.98 (95%CI: 0.98 - 0.99), respectively (Table 2). These mean that every one-unit (nmol/mg protein) increase in plasma protein carbonyl the risk for HCC (odds of being HCC) is increased 5.15 times and every one-unit (VCEAC, mg/L) increase in plasma TAC level the risk for HCC is reduced about 2% ((1 - 0.98) x 100). These findings indicated that plasma protein carbonyl and TAC were independent predictors of the development of HBV-associated HCC.

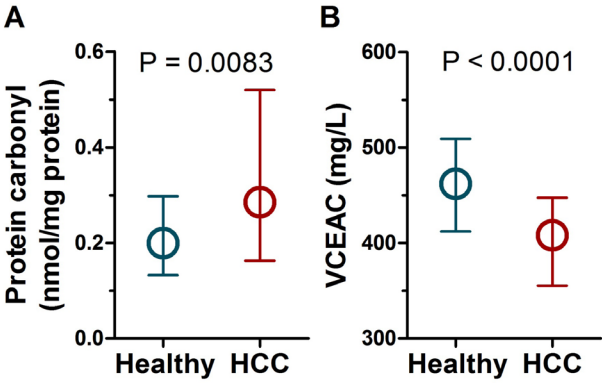

Figure 1. Plasma Protein Carbonyl Content and TAC in Healthy (n=80) and HBV-Associated HCC Subjects (n=60). Plasma levels of protein carbonyl and TAC (expressed as VCEAC) in HBV-associated patients were significantly higher than healthy controls. Data presented as median (IQR)

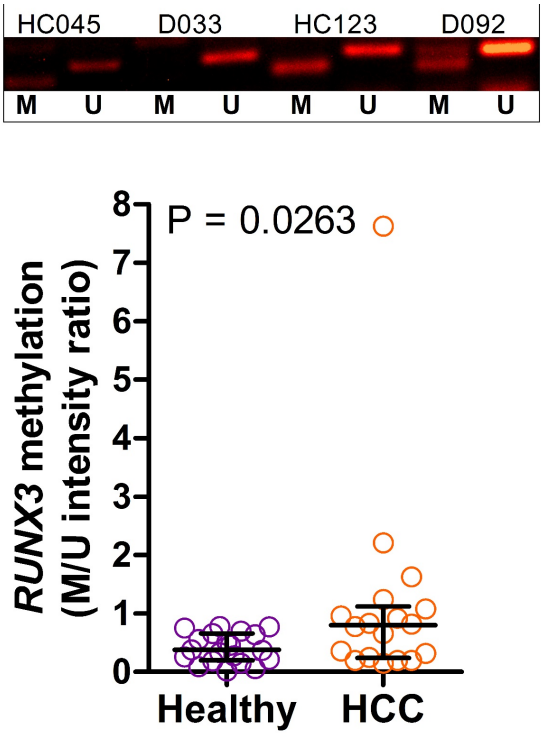

Figure 2. *RUNX3* Methylation Levels in PBMC Compared between Healthy (n=20) and HCC (n=18). Above panel: Representative gel of *RUNX3* methylation detection by MSP in two healthy subjects (D033 and D092) and two HCC patients (HC045 and HC123). As indicated by M/U intensity ratio, *RUNX3* methylation in PBMC of HCC patients was significantly higher than healthy individuals. Bars indicate medians and IQR. M: methylation (115 bp), U: unmethylation (124 bp)

Table 1. Characteristics of the Studied Subjects

Characteristics	Healthy	HBV-associated HCC	P value
Total number of subjects	80	60	
Age (years)	50.59±5.54	52.33±7.91	0.127
Males: Females (% male)	50:30 (62.50)	52:8 (86.67)	0.001
Cirrhosis (%)		52 (100)	
Yes		41 (78.85)	
No		11 (21.15)	
BCLC staging (%)		52 (100)	
0 (very early stage)		2 (3.85)	
A (early stage)		7 (13.46)	
B (intermediate stage)		19 (36.54)	
C (advanced stage)		24 (46.15)	

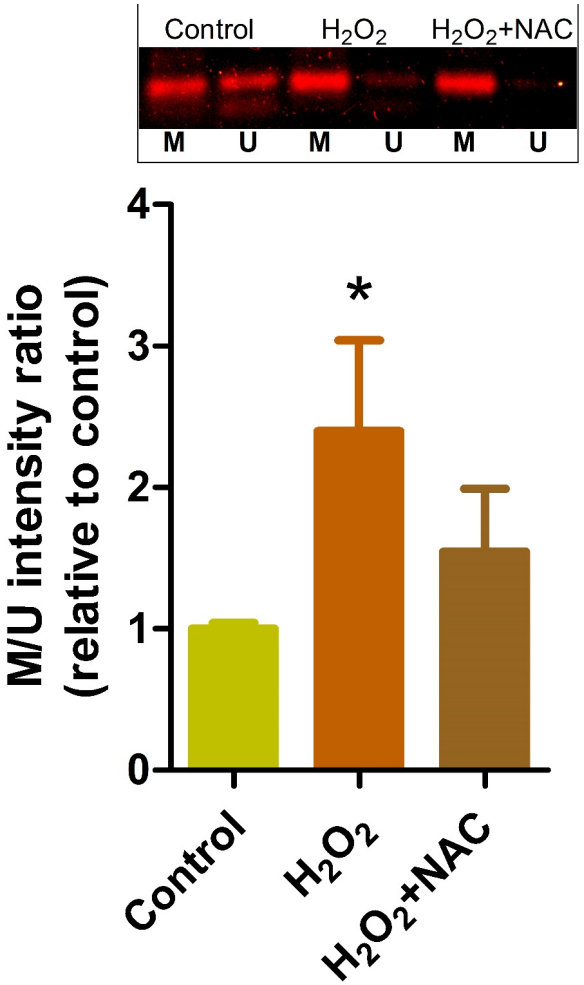

Figure 4. *RUNX3* Methylation Levels in HepG2 Cells Exposed to 20 μM H_2O_2 (Non-Lethal dose). Above panel: Representative gel of *RUNX3* methylation detection by MSP in untreated control cells, cells treated with 20 μM H_2O_2 and cells treated with 20 μM H_2O_2 plus 50 μM NAC. The M/U intensity ratio in cells treated with H_2O_2 was significantly greater than that in untreated control. *RUNX3* methylation in cells treated with H_2O_2 and NAC was lower than in cells treated with H_2O_2, although it was not statistically significant yet. Bars indicate standard errors. M: methylation, U: unmethylation. *P<0.05 *vs*. untreated control

Increased RUNX3 methylation in HBV-associated HCC patients

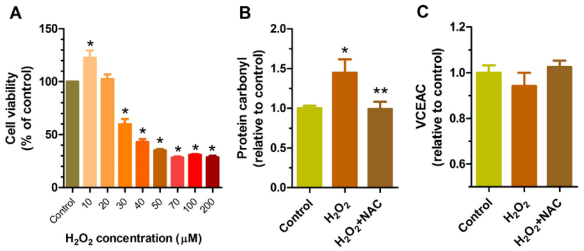

Figure 3. Cell Viability and Oxidative Stress in HepG2 Cells Treated with H2O2 for 72 h. A: % cell viability was compared among cells treated with different concentrations of H_2O_2. Cell survival significantly increased in cells treated with 10 μM H2O2, but significantly decreased in cells treated with 30 μM and higher concentrations of H_2O_2, as compared to the untreated control. No significant change of cell viability in cells treated with 20 μM H_2O_2 relative to control. B: Protein carbonyl content in cell lysate compared among untreated control, cells treated with 20 μM H_2O_2, and cells treated with 20 μM H_2O_2 and 50 μM NAC. Protein carbonyl content was significantly increased in cells treated with 20 μM H_2O_2 compared with the untreated cells. Co-treatment with NAC significantly decreased protein carbonyl formation in the H_2O_2-treated cells. C: TAC in the H_2O_2-treated cells trended to be decreased relative to the control, and co-treatment with NAC caused restoration of antioxidant capacity, although statistically significant difference was not revealed. *P<0.05 vs. untreated control. **P<0.05 vs. H_2O_2-treated cells. Bars: means, error bars: standard errors

Of 140 subjects, 38 had PBMC DNA available for determination of *RUNX3* methylation. Based on M/U intensity ratio presented herein, patients with HCC (n=18) had significantly increased *RUNX3* methylation, as compared to the healthy individuals (n=20) (Figure 2). Mean age (52.39 *vs*. 52.55 years) and sex distribution (85.00% *vs*. 94.44% females) between the HCC and control groups were not significantly different. Representative gel for *RUNX3* methylation detection by MSP is shown in Figure 2.

Induction of RUNX3 hypermethylation by H_2O_2 in HepG2 cell line

Causal relationship between oxidative stress and *RUNX3* hypermethylation was investigated in cell culture model. H_2O_2 was used as representative of ROS to stimulate oxidative stress in HepG2 cells (Figure 3). MTT assay showed that treatment with 10 μM H_2O_2 for 72 h significantly increased cell survival, but treatments

Table 2. Logistic Regression Measuring Association Strength of Plasma Protein Carbonyl and Total Antioxidant Capacity with HCC Adjusted for Age and Gender

Variables	Adj. OR* (coeff.)	SE	P value	95%CI
Plasma protein carbonyl (nmol/mg protein)	5.15 (1.64)	2.877	0.003	3.68-14.20
Total antioxidant capacity, VCEAC (mg/L)	0.98 (-0.02)	0.004	< 0.001	0.98-0.99

*Adj. OR: adjusted odds ratio, coeff.: β coefficient, SE: standard error, CI: confidence interval, VCEAC: vitamin C equivalent antioxidant capacity.

with 30 μM and more concentrations of H_2O_2 gradually caused significant decrease in cell viability (Figure 3A). HepG2 cells treated with 20 μM H_2O_2 for 72 h did not alter cell viability. Therefore, we opted to use the non-lethal concentration of H_2O_2 at 20 μM for investigating the effect of ROS on *RUNX3* methylation in HCC cells.

Cells treated with 20 μM H_2O_2 caused significantly increased in cellular protein carbonyl content, as compared to the untreated control cells (Figure 3B). Co-treatment with NAC caused a significant decrease in protein carbonyl content, as compared to the H_2O_2-treated cells. Although significant differences were not revealed yet, cellular TAC trended to be decreased in the H_2O_2-treated cells and trended to be restored in the cells co-treated with H_2O_2 and NAC (Figure 3C). These indicated an increase in oxidative stress in HepG2 cells challenged with 20 μM H_2O_2.

RUNX3 M/U ratio in H_2O_2-treated HepG2 cells was significantly higher than the untreated controls (Figure 4). The M/U ratio trended to be decreased in cells co-treated with H_2O_2 and NAC, although significant difference was not observed yet. Representative gel image compared among these three cultured conditions is shown in Figure 4.

Discussion

Chronic hepatitis infection either with HBV or HCV is a primary etiologic cause of HCC. Mechanistic insight into hepatocarcinogenesis reveals that oxidative stress promotes both genetic mutation and epigenetic alteration (Nishida and Kudo, 2013). Several lines of evidences demonstrate an increase in oxidative stress in HBV-associated HCC (Tsai et al., 2009, Nair et al., 2010, Zhao et al., 2011). *RUNX3* hypermethylation is also frequently detected in the HCC tissues (Yang et al., 2014, Zhang et al., 2015), suggesting a vital role in the HCC genesis. To date, the mechanism of how *RUNX3* is hypermethylated in the HCC is not known. In this study, we demonstrated an increased oxidative stress and hypermethylation of *RUNX3* promoter in patients with HBV-associated HCC. We additionally showed that increased extent of oxidative stress was independently associated with an increased risk for HCC development. Importantly, we experimentally demonstrated that H2O2 was capable of inducing *RUNX3* hypermethylation in HepG2 cells, indicated that ROS was an inducer of *RUNX3* hypermethylation.

Oxidative stress-induced DNA methylation alteration in cancers gains more and more recognition (Nishida and Kudo, 2013, Wu and Ni, 2015). ROS-induced promoter hypermethylation of TSG in HCC is well demonstrated for E-cadherin (Lim et al., 2008). The authors conclude that Snail expression induced by H2O2 leads to recruiting histone deacetylase 1 (HDAC1) and DNA methyltransferase 1 (DNMT1), which subsequently causes hypermethylation of E-cadherin promoter. Similar mechanism that H_2O_2 induces HDAC1 and DNMT1 expressions leading to hypermethylation of tumor suppressor caudal type homeobox-1 is also demonstrated in the colorectal cancer cells (Zhang et al., 2013). Additionally, increased expression and activity of HDAC1 and DNMT1 at the *RUNX3* promoter resulting in *RUNX3* hypermethylation is shown in the colon cancer cell lines challenged with H_2O_2 (Kang et al., 2012). Whether the ROS-induced *RUNX3* hypermethylation in HCC cells is mediated via this mechanism remains to be elucidated.

The other mechanism for ROS-induced DNA hypermethylation is that H_2O_2 induces the formation of a large silencing complex comprising of DNMT1, histone deacetylase SIRT1 and polycomb repressive complex 4 (O'Hagan et al., 2011). Such a large silencing complex relocalizes from non-GC-rich to GC-rich regions including promoter CpG islands, which in turn causes hypermethylation of the CpG-rich promoters. In this study, we clearly showed in HCC cells that H_2O_2 is an inducer of *RUNX3* hypermethylation, however the mechanism is unknown. Further study is awaiting to conduct to uncover the molecular mechanism of *RUNX3* hypermethylation induced by ROS in HCC. Since *RUNX3* hypermethylation is associated with HCC progression, and loss of *RUNX3* is shown to induce EMT in the low-metastatic HCC cells (Tanaka et al., 2012), treatment with antioxidants might reestablish the unmethylated state of *RUNX3*, which in turn leads to re-expression of *RUNX3* and deceleration of the tumor progression.

Limitations of the current study should be mentioned. We did not have data of oxidative stress and *RUNX3* hypermethylation in HCC tissues to evaluate if they corresponded well with the measurements in blood samples. Samples size for detecting PBMC *RUNX3* methylation was rather small. The transcript expression of *RUNX3* did not measured. One cell line was investigated to demonstrate an induction of *RUNX3* hypermethylation by ROS. The dose-dependent fashion of ROS-induced hypermethylation did not explored.

In conclusion, to the authors' knowledge this is the first study demonstrating an increase in oxidative stress coincided with hypermethylation of *RUNX3* in patients with HBV-associated HCC. Increased degree of oxidative stress is an independent predictor for HCC development. It is also shown for the first time that ROS is able to induce the *RUNX3* hypermethylation in HCC cells indicating a cause-and-effect relationship between oxidative stress and hypermethylation of *RUNX3* promoter. Antioxidant

regimen might be beneficial to restore the unmethylated state of *RUNX3*, in order to re-express this tumor suppressor protein in the HBV-related liver cancer.

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