RESEARCH ARTICLE

Epigenetic changes induced by oxidative stress in colorectal cancer cells: methylation of tumor suppressor RUNX3

Kyoung Ah Kang · Rui Zhang · Gi Young Kim · Suk Chul Bae · Jin Won Hyun

Abstract Runt domain transcription factor 3 (RUNX3) is a tumor suppressor that is silenced in cancer via hypermethylation of its promoter. This study investigated the mechanisms involved in reactive oxygen species (ROS)-induced silencing of RUNX3 in terms of epigenetic alteration since the effects of oxidative stress in tumor suppressor gene transcription are largely unknown. RUNX3 mRNA and protein expressions were down-regulated in response to hydrogen peroxide (H_2O_2) in the human colorectal cancer cell line SNU-407. This down-regulation was abolished with pretreatment of the ROS scavenger, N-acetylcysteine (NAC). Moreover, methylation-specific PCR data revealed that H_2O_2 treatment increased RUNX3 promoter methylation; however, NAC and the cytosine methylation inhibitor, 5-aza-2-deoxycytidine (5-Aza-dC), decreased it, suggesting that an epigenetic regulatory mechanism by ROS-induced methylation may be involved in RUNX3 silencing. H_2O_2 treatment resulted in DNA methyltransferase 1 (DNMT1) and histone deacetylase 1 (HDAC1) up-regulation with increased expression and activity, increased binding of DNMT1 to HADC1, and increased DNMT1 binding to the RUNX3 promoter. In addition, 5-Aza-dC treatment prevented the decrease in RUNX3 mRNA and protein levels by H_2O_2 treatment. Additionally, H_2O_2 treatment inhibited the nuclear localization and expression of RUNX3, which was abolished by NAC treatment. Furthermore, the down-regulation of RUNX3 expression by H_2O_2 also influenced cell proliferation. Taken together, the data suggested that ROS silenced the tumor suppressor, RUNX3, by epigenetic regulation and may therefore be associated with the progression of colorectal cancer.

Keywords Runt domain transcription factor 3 · Tumor suppressor gene · Colorectal cancer · Reactive oxygen species · Epigenetic alteration

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Introduction

It has been suggested that reactive oxygen species (ROS) participate in tumor progression by promoting DNA damage and/or altering cellular signaling pathways [1]. ROS are known to modulate growth signals and to regulate gene expression, leading to the sustained proliferation of cancer cells [2]. DNA methylation is the most frequent epigenetic alteration seen in mammalian genomes, and it often mediates transcriptional repression [3, 4]. Recently, evidence has emerged that both genetic and epigenetic changes underlie carcinogenesis [5] and, in particular, hypermethylation of CpG islands in promoter regions of tumor suppressor genes has been commonly observed in various cancers [6, 7].

Oxidative stress due to metabolic, dietary, and environmental factors leads to the excessive production of ROS. ROS production is associated with increased DNA damage including base modifications, deletions, strand breakage, and chromosomal rearrangements [8, 9] and chromosomal alteration with both hyper- and hypomethylation of DNA [10].

The runt-domain transcription factor 3 (RUNX3) is known to be a tumor suppressor involved in various cancers, including gastric cancer [11–14]. RUNX3 is necessary for gastric epithelial growth [15], neurogenesis of dorsal root ganglia [16, 17], and T-cell differentiation [18], and RUNX3 knockout mice exhibited gastric epithelial hyperplasia, reduced apoptosis, and sensitivity to transforming growth factor (TGF)-β [15]. Approximately 45% to 60% of human gastric cancers were reported to display loss of RUNX3 expression due to hemizygous deletion and promoter hypermethylation [15], while chronic gastritis and gastric adenomas showed RUNX3 hypermethylation, indicating a role for RUNX3 in gastric cancer progression [19]. Thus, an evaluation of the RUNX3 methylation status in normal gastric mucosa may provide an estimate of the risk for gastric carcinogenesis [20]. Besides hypermethylation, it was reported that RUNX3 inactivation also occurs in response to the mislocalization of the RUNX3 protein from the nucleus to the cytoplasm [21–25]. Ito et al. showed that when the RUNX3 protein was translocated to the nucleus in response to TGF-β, it acted as tumor suppressor; however, cytoplasmic RUNX3 protein did not elicit tumor suppressive activity, suggesting that it had been inactivated [21]. The gastrointestinal tract, especially the colon and rectum, is constantly exposed to ROS originating from endogenous and exogenous sources [26]. A role for ROS in colorectal cancer remains speculative, but numerous epidemiological studies suggest that oxidative stress may be important in cancer initiation and progression [14, 27–30].

In this study, we investigated whether oxidative stress can regulate the expression of the tumor suppressor gene, RUNX3, in colorectal cancer cells. The data showed that RUNX3 silencing by oxidative stress is mediated by epigenetic DNA methylation and suggested that it may play a role during the development of colorectal cancer.

Materials and methods

Cell culture

Human colorectal cancer cell lines, HT-29, SW-403, SNU-407, SNU-1033, and Caco-2, from the Korean Cell Line Bank (Seoul, Republic of Korea) and normal human colon FHC cells from the American Type Culture Collection (Rockville, MD, USA) were maintained at 37°C in an incubator with a humidified atmosphere of 5% CO_2. HT-29, SW-403, SNU-

407, and SNU-1033 cells were cultured in RPMI 1640 medium containing 10% fetal calf serum (FCS), streptomycin (100 μg/ml), and penicillin (100 U/ml). Caco-2 cells were cultured in minimum essential medium containing 10% FCS, streptomycin, and penicillin. FHC cells were cultured in a a 1:1 mixture of Ham's F12 and Dulbecco's modified Eagle's medium containing HEPES (25 mM), cholera toxin (10 ng/ml), insulin (5 μg/ml), transferrin (5 μg/ml), hydrocortisone (100 ng/ml), and 10% FCS.

RNA isolation and reverse transcription-polymerase chain reaction

Total RNA was isolated from cells using Trizol reagent (GibcoBRL, Grand Island, NY, USA), and cDNA was amplified using 1 ml of the reverse-transcription reaction buffer, primers, dNTPs, and 0.5 U of Taq DNA polymerase in a final volume of 25 ml as described previously [31]. The polymerase chain reaction (PCR) conditions consisted of 5 min at 94°C for the initial denaturation, followed by 35 cycles of 1 min at 94°C, 1 min at 55°C, 1 min at 72°C, and a final elongation step of 7 min at 72°C. The following primers were used to amplify the RUNX3 cDNA [32]: sense, 5′-GGCAATGACGAGAACTAC-3′ (located in exon 2); antisense, 5′-GGAGAATGGGGTTCAGTTC-3′ (located in exon 5). The amplified products were resolved by 1% agarose gel electrophoresis, stained with ethidium bromide, and photographed under UV light using Image Quant™ TL analysis software (Amersham Bioscience, Sweden).

Western blot analysis

Nuclear extracts were prepared using a nuclear protein extraction kit (Cayman Chemical, Ann Arbor, MI, USA), and nuclear extracts were lysed on ice with 1 ml of lysis buffer (10 mM Tris–HCl, pH 7.9, 10 mM NaCl, 3 mM $MgCl_2$, and 1% NP-40) for 4 min. After centrifugation for 10 min at 3,000×g, the pellets were re-suspended in 50μl of extraction buffer (20 mM HEPES, pH 7.9, 20% glycerol, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 1 mM DTT, and 1 mM PMSF), incubated on ice for 30 min, and then centrifuged at 13,000×g for 5 min. The supernatants were stored at −70°C after determination of the protein concentration. Aliquots of the lysates (40 μg of protein) were boiled for 5 min and were electrophoresed on 10% SDS-polyacrylamide gel. Blots of the gels were transferred to nitrocellulose membranes, which were subsequently incubated with primary antibodies, and then with secondary immunoglobulin-G-horseradish peroxidase conjugates (Pierce, Rockford, IL, USA). Protein bands were detected using an enhanced chemiluminescence Western blotting detection kit (Amersham, Little Chalfont, Buckinghamshire, UK). The protein bands were visualized using a luminescent image analyzer.

Quantitative real-time PCR

cDNA synthesis was performed with 2 μg RNA using a Superscript kit (Invitrogen, Carlsbad, CA, USA). Quantitative real-time PCR was performed with 2× SYBR Green Mastermix (Invitrogen, Carlsbad, CA, USA) and 900 nM primers for RUNX3 and 18 s rRNA. The thermal cycling parameters were as follows: an initial cycle of Taq activation for 1 min at 94°C, 1 min at 55°C, and 1 min at 72°C, optimized annealing temperature 5 s at 58–60°C, 30 s at 72°C, and a detection step of 8 s at 80°C. Reactions were performed in duplicate and the specificity was monitored using melting curve analysis after cycling. The following primers were designed by Bioneer Co. Ltd. (Seoul, Republic of Korea) (5′–3′) 18 s rRNA, 5′-CAGCCACCCCAGATT GAGCA-3′ and 5′-TAGTAGCGACGGGCGGTGTG-3′; (5′–3′) RUNX3, 5′-GACAGCCCCAACTTCCTCT-3′ and 5′-CACAGTCACCACCGTACCAT-3′. All data were tested first for normality and data with a non-normal distribution were subjected to square root transformation prior to statistical analyses. The comparative cycle threshold (C_t) method was used to calculate the relative changes in gene expression in the iQ5 real-time PCR system (Bio-Rad Laboratories, Hercules, CA, USA). The 2-delta-delta C_t value was calculated after 18 s RNA normalization [33].

Methylation-specific PCR

DNA bisulfate modification was carried out with the Methylamp™ DNA modification kit (Epigentek, Pittsburgh, PA, USA) according to the manufacturer's instructions. To analyze RUNX3 DNA methylation, methylation-specific (MS)-PCR was performed using an Epitect MSP kit (Qiagen, Valencia, CA, USA). The methylated and unmethylated RUNX3 primer sets were as follows [15]: unmethylated RUNX3, 5′-TTAT GAGGGGTGGTTGTATGTGGG-3′ and 5′-AAAACAAC CAACACAAACACCTCC-3′; methylated RUNX3, 5′-TTACGAGGGGCGGTCGTACGCGGG-3′ and 5′-AAAAC GACCGACGCGAACGCCTCC-3′. The PCR products were separated on 6% non-denaturing polyacrylamide gels, stained with ethidium bromide, and visualized under UV light.

DNA methyltransferase activity

DNA methyltransferase (DNMT) activity in nuclear extract was detected using a colorimetric assay with the EpiQuik DNMT activity assay kit (Epigentek). In this assay, the cytosine-rich DNA substrate is stably coated on the strip wells, and DNMT transfers a methyl group from S-denosylmethionine to a cytosine in the DNA substrate. The methylated DNA can be recognized using a 5-methylcytosine antibody. The levels of methylated DNA, which are proportional to the enzymatic activity, were then colorimetrically quantified. The results were expressed in absorbance units at 450 nm and were represented as a percentage of the activity.

Histone deacetylase (HDAC) activity

Histone deacetylase (HDAC) activity in nuclear extract was measured using the EpiQuik HDAC activity assay kit (Epigentek). The nuclear extracts were incubated with a specific substrate for 1 h at 37°C, followed by the capture antibody for 60 min, and then the detection antibody for 30 min at room temperature. Absorbance was determined using a microplate spectrophotometer at 450 nm and was represented as a percentage of the activity.

Immunoprecipitation and Western blot for detection of HDAC1

DNMT1 was immunoprecipitated from the nuclear extracts using a DNMT1 antibody. Immunoprecipitated complex was collected with protein G beads and washed with immunoprecipitation buffer. Equal amounts of precipitates were run on SDS–polyacrylamide gel and Western blotting was performed with an HDAC1 antibody.

Chromatin immunoprecipitation assay

Chromatin immunoprecipitation (ChIP) assay was performed using the SimpleChIP™ enzymatic chromatin IP kit (Cell Signaling Technology, Danvers, MA, USA) according to the manufacturer's protocol with slight modifications. Cells were pretreated with 1 mM NAC for 1 h, with H_2O_2 treatment for 48 h and cross-linked by addition of 1% formaldehyde. Chromatin was prepared and digested with nuclease for 12 min at 37°C. ChIP assay was performed with a rabbit DNMT1 polyclonal antibody (Abcam, Cambridge, MA, USA) and normal rabbit IgG. The antibodies were added to the chromatin digests and incubated with constant rotation overnight at 4°C. ChIP-grade protein G magnetic beads were added to capture the immunoprecipitated complexes. The beads were washed and the immunoprecipitates eluted with ChIP elution buffer. The cross-links were reversed by incubation at 65°C for 30 min. Proteinase K was added and incubated at 65°C for 2 h. The immunoprecipitated DNA fragments were then purified using spin columns, and the DNA recovered from the immune-precipitated complex was subjected to PCR with 35 cycles. The promoter region of RUNX3 gene in ChIP assay exists between −1618 and −1307 of RUNX3 gene sequence. The primers for the RUNX3 gene promoter were: forward, 5′-GGTTGCAGAAGTCACAGG-3′; reverse, 5′-AATTTGCT TAGAACGTCCG-3′ [34, 35]. PCR products were separated on 2% agarose gels and DNA bands were visualized using the Image program (NIH, Bethesda, MD, USA).

Immunocytochemistry

Cells plated on coverslips were fixed in 4% paraformaldehyde for 30 min and permeabilized with 0.1% Triton X-100 in PBS for 2.5 min. Cells were treated with blocking medium (3% bovine serum albumin in PBS) for 1 h and incubated with RUNX3 antibody diluted in blocking medium for 2 h. The primary RUNX3 antibody was detected by a 1:500 dilution of the FITC-conjugated secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 1 h. After washing with PBS, the stained cells were mounted on microscope slides in the mounting medium with DAPI (Vector, Burlingame, CA, USA). Images were collected using the LSM 510 program on a Zeiss confocal microscope.

Colony formation assay

Cells were seeded into 60-mm dishes at a density to produce approximately 200 colonies per dish. After treatment of H_2O_2 at 100 μM, the cultures were further incubated for 10 days. The resultant colonies were fixed with 75% ethanol and 25% acetic acid and stained with trypan blue. The colonies containing 50 or more cells were considered viable.

Statistical analysis

All measurements were performed in triplicate and all values are represented as the mean ± standard error of the mean. The results were subjected to analysis of variance using Tukey's test to analyze differences. $P < 0.05$ was considered significant.

Results

RUNX3 expression in colorectal cancer cell lines

RUNX3 mRNA and protein expression levels were analyzed by RT-PCR and Western blotting in five colorectal cancer cell lines: HT-29, SW-403, SNU-407, SNU-1033, and Caco-2. High levels of RUNX3 expression were observed in all cell lines except for HT-29 (Fig. 1a, b). SNU-407, which showed the highest level of RUNX3 expression, was selected for further study.

ROS down-regulated RUNX3 expression in colorectal cancer cell lines

Cells were treated with 100 μM of H_2O_2 during the 48-h incubation period and H_2O_2 (100 μM), for 48 h, did not show a cytotoxic effect to the SNU-407 cells.
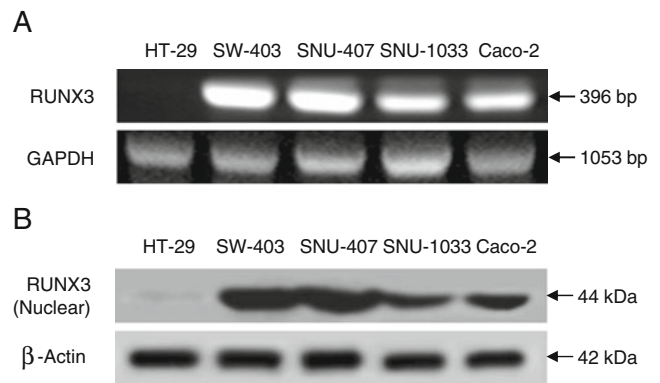
Fig. 1 Expression of RUNX3 in colorectal cancer cell lines. **a** RUNX3 mRNA level was analyzed by RT-PCR in the colorectal cancer cell lines, HT-29, SW-403, SNU-407, SNU-1033 and CaCo-2. **b** Nuclear extracts were electrophoresed and the cell lysates were immunoblotted using a RUNX3 antibody

However, H_2O_2 treatment produced a significant decrease in RUNX3 mRNA and protein levels as demonstrated by RT-PCR, real-time PCR, and Western blot analysis (Fig. 2a–c). NAC pretreatment in SNU-407 cells prevented the decreased RUNX3 expression by H_2O_2 treatment (Fig. 2d, e), suggesting that ROS are involved in regulating the tumor suppressor gene, RUNX3. In order to determine whether ROS-down-regulated RUNX3 expression is specific to SNU-407 cells, the mRNA and protein expression level of RUNX3 in SNU-1033 cells were also detected by RT-PCR, real-time PCR, and Western blot analysis. A similar pattern was observed in SNU-1033 cells, indicating that ROS-down-regulated RUNX3 expression is not unique to SNU-407 cells (Fig. 3).

ROS-induced methylation of the RUNX3 promoter

H_2O_2-treated cells showed methylation of the RUNX3 promoter as indicated by MS-PCR analysis, while pretreatment with the antioxidant, NAC, blocked H_2O_2-induced methylation (Fig. 4a). Pretreatment with the DNA methyltransferase inhibitor, 5-Aza-dC, decreased the level of RUNX3 promoter methylation induced by H_2O_2 treatment (Fig. 4b).

ROS-induced methylation-related proteins

H_2O_2-treated cells induced the expression and increased the activity of the methylation-related proteins, HDAC1 and DNMT1, in a time-dependent manner (Fig. 5a–c). In addition, H_2O_2 treatment increased the binding of HDAC1 to DNMT1 (Fig. 5d). The ChIP assay data demonstrated that H_2O_2 treatment increased the recruitment of DNMT1 to the RUNX3 promoter (Fig. 5e).
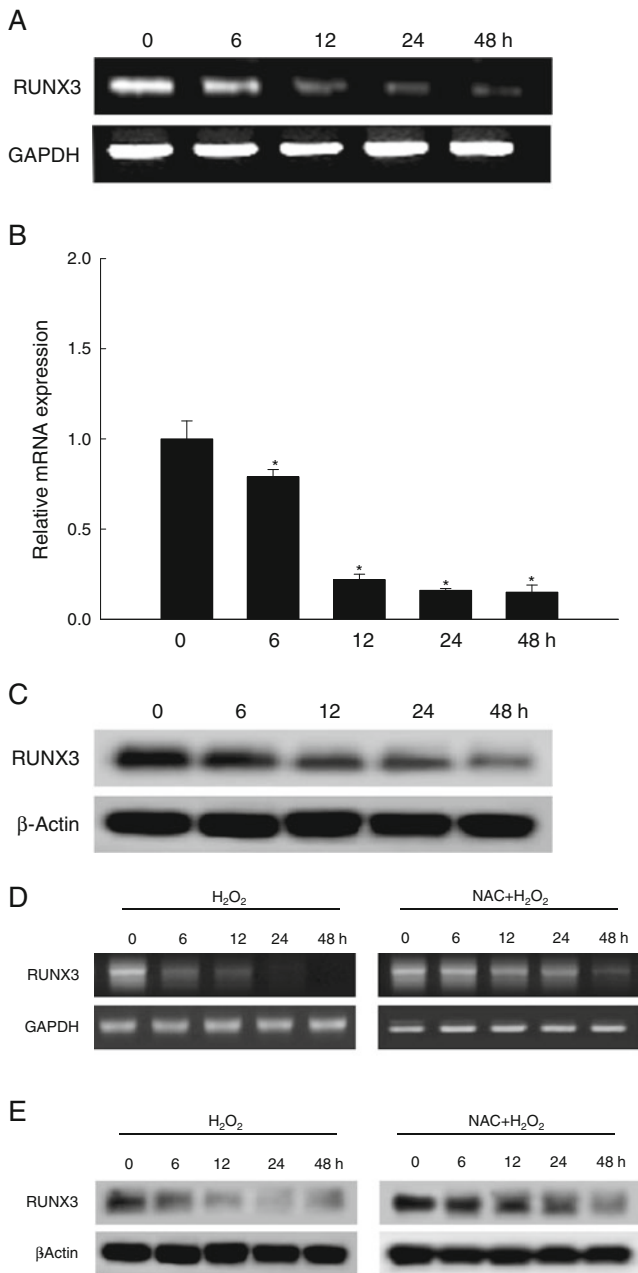
Fig. 3 ROS-down-regulated RUNX3 expression is not unique to SNU-407 cells. SNU-1033 cells were treated with 100 μM H_2O_2 during incubation for 48 h. Expression of RUNX3 following H_2O_2 treatment was assessed using **a** RT-PCR, **b** real-time PCR, and **c** Western blotting. *Asterisk* indicates significant difference from control ($p < 0.05$)

Fig. 2 Oxidative stress regulates RUNX3 expression in SNU-407 cells. Cells were treated with 100 μM H_2O_2 during incubation for 48 h. Expression of RUNX3 following H_2O_2 treatment was assessed using **a** RT-PCR, **b** real-time PCR, and **c** Western blotting. *Asterisk* indicates significant difference from control ($p < 0.05$). Cells were pre-treated with 1 mM NAC and 1 h later 100 μM of H_2O_2 was added and cells were incubated for 48 h. RUNX3 mRNA and protein expression were assessed using **d** RT-PCR and **e** Western blotting

Fig. 4 Oxidative stress induces methylation of the RUNX3 Promoter. Cells were pretreated with **a** 1 mM NAC or **b** 10 μM 5-Aza-dC and, 1 h later, exposed to H_2O_2 for 48 h. MS-PCR analysis of RUNX3 was performed on genomic DNA isolated from SNU-407 cells. Methylation was detected by the presence of a PCR product amplified by methylation-specific primers. Demethylation was detected by the presence of a PCR product amplified with unmethylation-specific primers. *M* methylated, *U* unmethylated DNA

Cytosine methylation inhibitor prevented the reduction in RUNX3 expression due to ROS

5-Aza-dC treatment prevented the levels of RUNX3 mRNA and protein reduced by H_2O_2 treatment (Fig. 6a, b). These
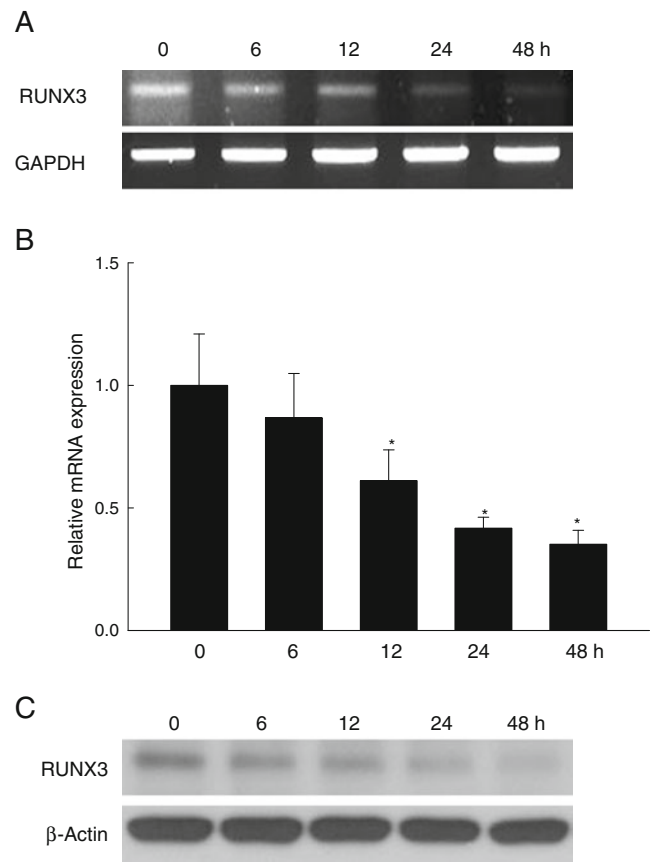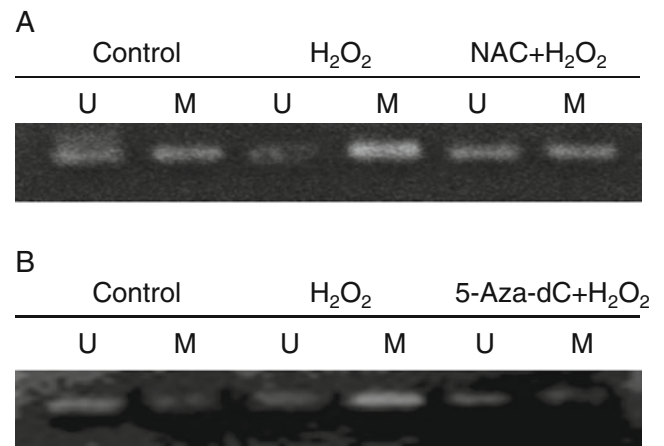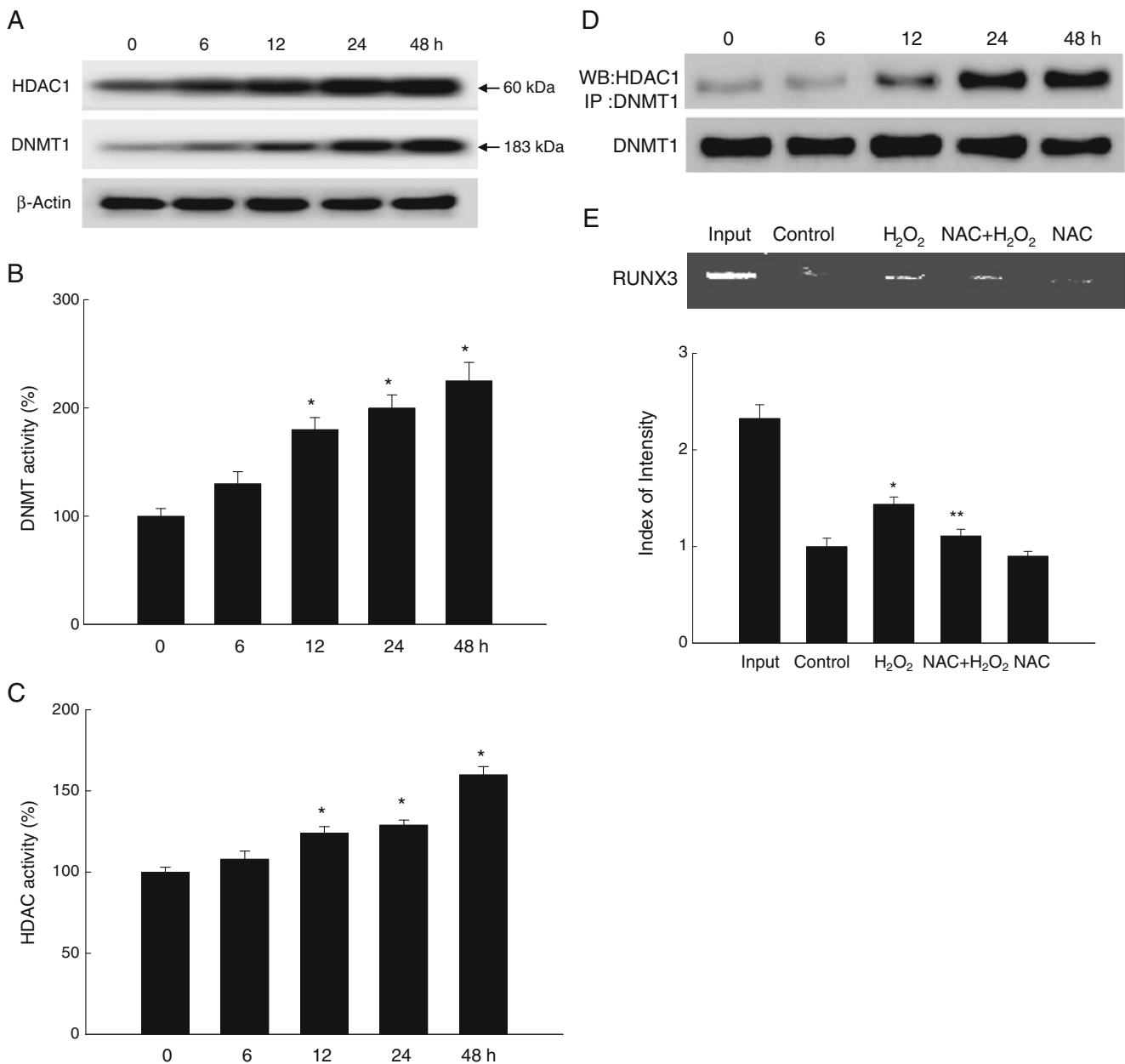
Fig. 5 Oxidative stress induces methylation of the RUNX3 promoter by activation of methylation-related proteins. **a** Western blotting analysis was performed with HDAC1 and DNMT1 antibodies. **b** DNMT activity was detected using a DNMT activity assay kit. *Asterisk* indicates significant difference from control ($p < 0.05$). **c** HDAC activity was measured using the HDAC activity assay kit. *Asterisk* indicates significant difference from control ($p < 0.05$). **d** Cell lysates were immunoprecipitated with a DNMT1 antibody, and precipitates were Western-blotted with a HDAC1 antibody. **e** ChIP analysis was performed with a DNMT1 antibody and primers amplifying the RUNX3 promoter region; the band shows the level of DNMT1 in the RUNX3 promoter region. *Input* represents amplification of total DNA from whole cell lysate. DNMT1 level in the RUNX3 promoter region was quantified and presented as an index of the intensity. *Asterisk* indicates significant difference from control ($p < 0.05$) and *two asterisks* indicate significant difference from H_2O_2 treatment ($p < 0.05$)

results suggested that oxidative stress decreased the expression of the tumor suppressor gene, RUNX3, via DNA methylation, and that this epigenetic change is associated with RUNX3 inactivation.

ROS-induced inactivation of RUNX3 by mislocalization

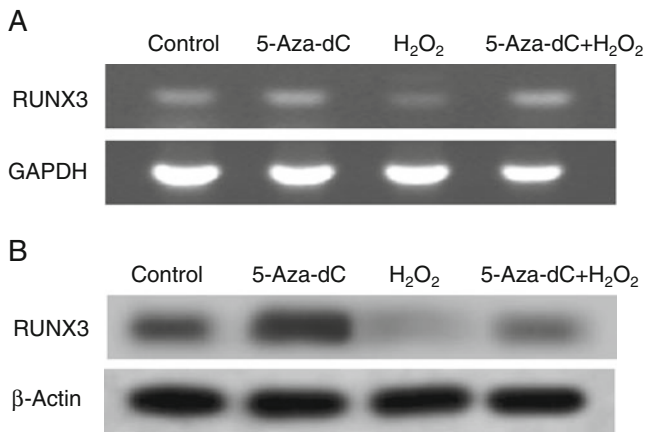The mislocalization of the nuclear RUNX3 protein to the cytoplasm was observed initially in gastric cancers [21],

Fig. 6 Effect of a DNA methylation inhibitor on RUNX3 expression under oxidative stress. Cells were pretreated with 10 μM 5-Aza-dC and, 1 h later, exposed to H$_2$O$_2$ for 72 h. **a** The RUNX3 mRNA expression was assessed using RT-PCR and **b** RUNX3 protein expression was analyzed by Western blotting

breast cancers [22, 23], colorectal polyps [24], and colorectal cancers [25, 36]. As shown in Fig. 7a, b, H$_2$O$_2$-treated cells exhibited increased levels of RUNX3 protein in the cytosol and decreased levels in the nucleus, while NAC pretreatment reduced the loss of RUNX3 from nucleus to cytosol in H$_2$O$_2$-treated cells, indicating that H$_2$O$_2$ treatment resulted in the mislocalization of RUNX3 from the nucleus to cytosol.

The influence of down-regulation of RUNX3 expression by ROS

To investigate whether the decrease in RUNX3 expression by ROS could influence cell phenotype, cell proliferation rate was examined by colony formation. As shown in Fig. 8a, b, H$_2$O$_2$ significantly down-regulated RUNX3 expression and increased cell proliferation. To further investigate whether the decrease in RUNX3 expression by ROS is reversible, cells were treated with 100 μM H$_2$O$_2$ for 24 h, and then the culture medium was changed. After 48 h, RUNX3 expression was detected by Western blotting. H$_2$O$_2$ eventually suppressed RUNX3 expression, indicating that H$_2$O$_2$-induced down-regulation of RUNX3 is irreversible (Fig. 8c).

Discussion

An imbalance in the production/removal of ROS can be either directly or indirectly involved in the initiation, promotion, and progression phases of carcinogenesis [37]. ROS may cause damage to DNA and chromosomes, induce epigenetic alterations such as hypermethylation of DNA or histone modification, interact with oncogenes or tumor suppressor genes, and impart changes to immunological

mechanisms [10, 38, 39]. The tumor suppressor, RUNX3, is frequently inactivated in gastric cancer tissues [15, 21] and its aberrant activity is closely related to metastatic outcome [40]. It has been postulated that the mechanism for RUNX3 inactivation in cancer cells and tissues involves DNA hypermethylation in the promoter region [15, 41]. Abnormal de novo methylation of CpG islands occurs frequently in various cancers, including colorectal cancers; moreover, hypermethylation of CpG islands is associated with transcriptional
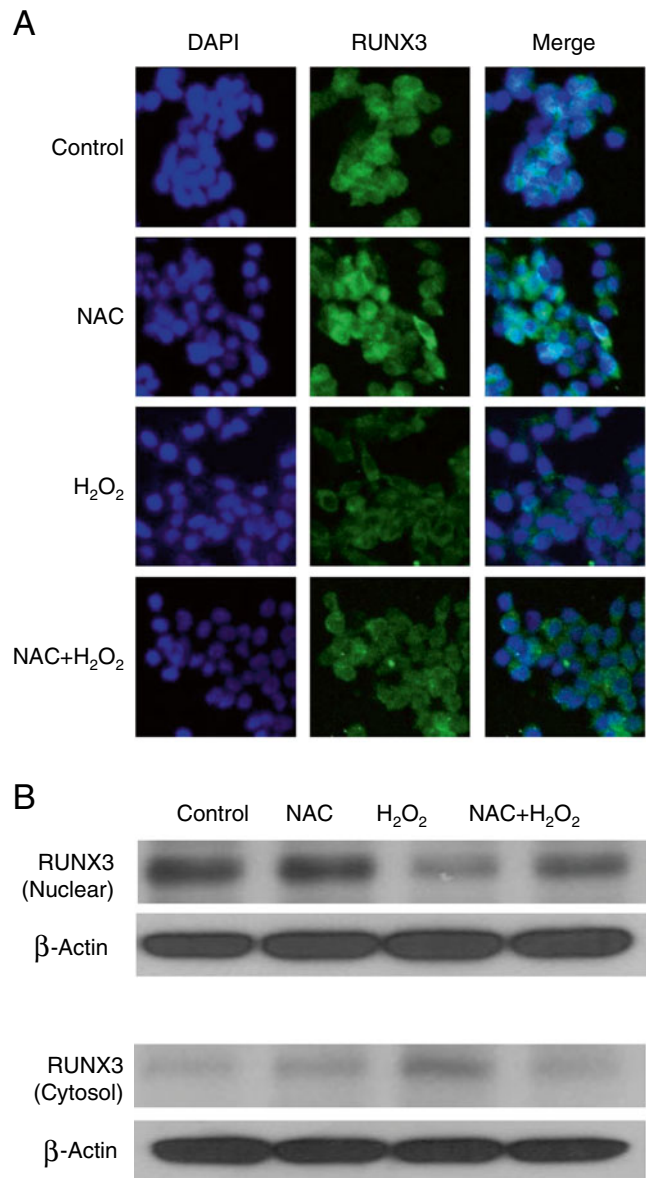

Fig. 7 Oxidative stress induces mislocalization of RUNX3. Cells were pretreated with 1 mM NAC and, 1 h later, exposed to H$_2$O$_2$ for 48 h. **a** Confocal images of RUNX3 antibody and FITC-conjugated secondary antibody staining indicate the location of RUNX3 protein (*green*); DAPI staining indicates the nucleus (*blue*). The merged images of H$_2$O$_2$-treated cells reveal the cytosolic location of RUNX3 protein. **b** Nuclear and cytoplasmic fractions were extracted and quantified. Extracts were electrophoresed and immunoblotted using a RUNX3 antibody
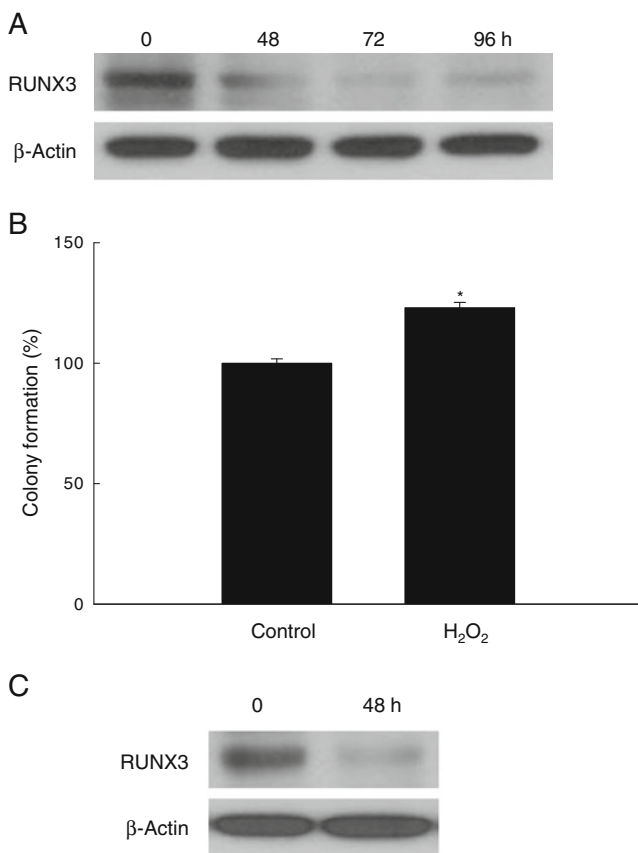
Fig. 8 The influence of down-regulation of RUNX3 expression by ROS on cell proliferation. **a** Cells were treated with 100 μM H_2O_2 for the indicated time and RUNX3 expression was assessed by Western blotting. **b** After treatment with H_2O_2, cells were allowed to grow for 10 days. The resulting colonies were stained with trypan blue and counted. *Asterisk* indicates significant difference from control ($p<0.05$). **c** Cells were treated with 100 μM H_2O_2 for 24 h, and then the culture medium was changed. After 48 h, RUNX3 expression was detected by Western blotting

inactivation [42–50]. The RUNX3 gene is located on human chromosome 1p36 [15], a region in which many genes play roles in the maintenance of chromosome stability, suppression of tumorigenesis, control of apoptosis, and DNA methylation [51, 52]. Deletions in the 1p36 region are common in colorectal cancers [51–55], suggesting that gene loss in this region might be implicated in chromosome instability [52].

In our study, all of the human colorectal cancer cell lines SW-403, SNU-407, SNU-1033, and Caco-2, except for HT-29, showed expression of RUNX3 mRNA and protein. A previous report confirmed that the RUNX3 promoter was methylated and RUNX3 was not expressed in the cell line HT-29 [32]. Our data showed that RUNX3 expression was down-regulated in response to H_2O_2 treatment in SNU-407 cells and that this down-regulation was abolished with the ROS scavenger, NAC. MS-PCR data revealed that oxidative stress affected the methylation of the RUNX3 promoter and that treatment with the DNA methylation inhibitor, 5-Aza-dC, resulted in the re-expression of RUNX3, suggesting that

an epigenetic regulatory mechanism may be involved in RUNX3 silencing by ROS-induced methylation. DNA methylation catalyzed by DNMTs and histone deacetylation catalyzed by HDACs play an important role in the regulation of gene expression during carcinogenesis. Considerable evidence has accumulated in the elucidation of the molecular mechanisms in which DNA methylation is involved in tumor suppressor gene silencing [56].

The methylation of CpG dinucleotides is catalyzed by DNMTs, which transfer the methyl moiety from the methyl donor, S-adenosylmethionine, to the 5′ position of the cytosine ring. DNMT1 has a preference for methylating hemimethylated DNA and is referred to as the primary maintenance DNMT [57, 58]. HDAC and DNMT1 cooperate to initiate and sustain epigenetic gene silencing [59]. In vivo studies have shown that DNMT1 associates with HDAC1 to deacetylate chromatin and silence gene transcription [60, 61]. In support of these data, our results showed that H_2O_2 treatment resulted in DNMT1 and HDAC1 up-regulation with increased expression, increased activity, increased binding of DNMT1 to HADC1, and increased DNMT1 binding to the RUNX3 promoter. In addition, 5-Aza-dC treatment prevented the decrease in RUNX3 mRNA and protein levels by H_2O_2 treatment. The mislocalization of active nuclear RUNX3 protein to the cytoplasm has been observed in various cancers [21–26]. In the present study, H_2O_2 treatment inhibited the nuclear localization and expression of RUNX3, and this inhibition was abolished by NAC pretreatment.

Taken together, the data presented in this study suggest that oxidative stress may play an important role in inhibiting the activation of the tumor suppressor, RUNX3, in human colorectal cancer cells, via promoter methylation, and that this epigenetic regulation may be associated with the progression of colorectal cancer.

References

1. Hussain SP, Hofseth LJ, Harris CC. Radical causes of cancer. Nat Rev Cancer. 2003;3:276–85.
2. Filomeno G, Rotilio G, Ciriolo MR. Disulfide relays and phosphorylative cascades: partners in redox-mediated signaling pathways. Cell Death Differ. 2005;12:1555–63.
3. Ballestar E, Paz MF, Valle L, Wei S, Fraga MF, Espada J, et al. Methyl-CpG binding proteins identify novel sites of epigenetic inactivation in human cancer. EMBO J. 2003;22:6335–45.
4. Cedar H. DNA methylation and gene activity. Cell. 1988;53:3–4.
5. Jones PA, Baylin SB. The epigenomics of cancer. Cell. 2007;128:683–92.
6. Esteller M. CpG island hypermethylation and tumor suppressor genes: a booming present, a brighter future. Oncogene. 2002;21:5427–40.

7. Herman JG, Baylin SB. Gene silencing in cancer in association with promoter hypermethylation. N Engl J Med. 2003;349:2042–54.

8. Valko M, Izakovic M, Mazur M, Rhodes CJ, Telser J. Role of oxygen radicals in DNA damage and cancer incidence. Mol Cell Biochem. 2004;266:37–56.

9. Valko M, Rhodes CJ, Moncol J, Izakovic M, Mazur M. Free radicals, metals and antioxidants in oxidative stress-induced cancer. Chem Biol Interact. 2006;160:1–40.

10. Lim SO, Gu JM, Kim MS, Kim HS, Park YN, Park CK, et al. Epigenetic changes induced by reactive oxygen species in hepatocellular carcinoma: methylation of the E-cadherin promoter. Gastroenterology. 2008;135:2128–40.

11. Bae SC, Choi JK. Tumor suppressor activity of RUNX3. Oncogene. 2004;23:4336–40.

12. Hiramatsu T, Osaki M, Ito Y, Tanji Y, Tokuyasu N, Ito H. Expression of RUNX3 protein in human esophageal mucosa and squamous cell carcinoma. Pathobiology. 2005;72:316–24.

13. Oshimo Y, Oue N, Mitani Y, Nakayama H, Kitadai Y, Yoshida K, et al. Frequent loss of RUNX3 expression by promoter hypermethylation in gastric carcinoma. Pathobiology. 2004;71:137–43.

14. Subramaniam MM, Chan JY, Yeoh GK, Quek T, Ito K, Salto-Tellez M. Molecular pathology of RUNX3 in human carcinogenesis. Biochim Biophys Acta. 2009;1796:315–31.

15. Li QL, Ito K, Sakakura C, Fukamachi H, Inoue K, Chi XZ, et al. Causal relationship between the loss of RUNX3 expression and gastric cancer. Cell. 2002;109:113–24.

16. Inoue K, Ozaki S, Shiga T, Ito K, Masuda T, Okado N, et al. Runx3 controls the axonal projection of proprioceptive dorsal root ganglion neurons. Nat Neurosci. 2002;5:946–54.

17. Levanon D, Bettoun D, Harris-Cerruti C, Woolf E, Negreanu V, Eilam R, et al. The Runx3 transcription factor regulates development and survival of TrkC dorsal root ganglia neurons. EMBO J. 2002;21:3454–63.

18. Taniuchi I, Osato M, Egawa T, Sunshine MJ, Bae SC, Komori T, et al. Differential requirements for Runx proteins in CD4 repression and epigenetic silencing during T lymphocyte development. Cell. 2002;111:621–33.

19. Kim TY, Lee HJ, Hwang KS, Lee M, Kim JW, Bang YJ, et al. Methylation of RUNX3 in various types of human cancers and premalignant stages of gastric carcinoma. Lab Invest. 2004;84:479–84.

20. Nakase Y, Sakakura C, Miyagawa K, Kin S, Fukuda K, Yanagisawa A, et al. Frequent loss of RUNX3 gene expression in remnant stomach cancer and adjacent mucosa with special reference to topography. Br J Cancer. 2005;92:562–9.

21. Ito K, Liu Q, Salto-Tellez M, Yano T, Tada K, Ida H, et al. RUNX3, a novel tumor suppressor, is frequently inactivated in gastric cancer by protein mislocalization. Cancer Res. 2005;65:7743–50.

22. Lau QC, Raja E, Salto-Tellez M, Liu Q, Ito K, Inoue M, et al. RUNX3 is frequently inactivated by dual mechanisms of protein mislocalization and promoter hypermethylation in breast cancer. Cancer Res. 2006;66:6512–20.

23. Subramaniam MM, Chan JY, Soong R, Ito K, Ito Y, Yeoh KG, et al. RUNX3 inactivation by frequent promoter hypermethylation and protein mislocalization constitute an early event in breast cancer progression. Breast Cancer Res Treat. 2009;113:113–21.

24. Subramaniam MM, Chan JY, Soong R, Ito K, Yeoh KG, Wong R, et al. RUNX3 inactivation in colorectal polyps arising through different pathways of colonic carcinogenesis. Am J Gastroenterol. 2009;104:426–36.

25. Ito K, Lim AC, Salto-Tellez M, Motoda L, Osato M, Chuang LS, et al. RUNX3 attenuates beta-catenin/T cell factors in intestinal tumorigenesis. Cancer Cell. 2008;14:226–37.

26. Blau S, Rubistein A, Bass P, Singaram C, Kohen R. Differences in the reducing power along the rat GI tract: lower antioxidant capacity of the colon. Mol Cell Biochem. 1999;194:185–91.

27. Hendricks CW, Kelly RW, Radley S, Donovan IA, Keighley MR, Neoptolemis JP. Lipid peroxidation and prostaglandins in colorectal cancer. British J of Surg. 1994;81:1219–23.

28. Van Driel BE, Lyon H, Hoogenraad DC, Anten S, Hansen U, Van Noorden CJ. Expression of CuZn- and Mn-superoxide dismutase in human colorectal neoplasms. Free Radic Biol Med. 1997;23:435–44.

29. Benhar M, Engelberg D, Levitzki A. ROS, stress-activated kinases and stress-signaling in cancer. EMBO Reports. 2002;3:420–5.

30. Skrzydlewska E, Sulkowski S, Koda M, Zalewski B, Kanczuga-Koda L, Sulkowska M. Lipid peroxidation and antioxidant status in colorectal cancer. World J Gastroenterol. 2005;11:403–6.

31. Chen JC, Huang KC, Lin WW. HMG-CoA reductase inhibitors upregulate heme oxygenase-1 expression in murine RAW264.7 macrophages via ERK, p38 MAPK and protein kinase G pathways. Cell Signal. 2006;18:32–9.

32. Ku JL, Kang SB, Shin YK, Kang HC, Hong SH, Kim IJ, et al. Promoter hypermethylation downregulates RUNX3 gene expression in colorectal cancer cell lines. Oncogene. 2004;23:6736–42.

33. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods. 2001;25:402–8.

34. Lee SH, Kim J, Kim WH, Lee YM. Hypoxic silencing of tumor suppressor RUNX3 by histone modification in gastric cancer cells. Oncogene. 2009;28:184–94.

35. Kondo Y, Shen L, Issa JP. Critical role of histone methylation in tumor suppressor gene silencing in colorectal cancer. Mol Cell Biol. 2003;23:206–15.

36. Soong R, Shah N, Peh BK, Chong PY, Ng SS, Zeps N, et al. The expression of RUNX3 in colorectal cancer is associated with disease stage and patient outcome. Br J Cancer. 2009;100:676–9.

37. Kang DH. Oxidative stress, DNA damage, and breast cancer. AACN Clinical Issues. 2002;13:540–9.

38. Arsova-Sarafinovska Z, Eken A, Matevska N, Erdem O, Sayal A, Savaser A, et al. Increased oxidative/nitrosative stress and decreased antioxidant enzyme activities in prostate cancer. Clin Biochem. 2009;42:1228–35.

39. Lawless MW, O'Byrne KJ, Gray SG. Oxidative stress induced lung cancer and COPD: opportunities for epigenetic therapy. J Cell Mol Med. 2009;13:2800–21.

40. Fahrner JA, Eguchi S, Herman JG, Baylin SB. Dependence of histone modifications and gene expression on DNA hypermethylation in cancer. Cancer Res. 2002;62:7213–8.

41. Li QL, Kim HR, Kim WJ, Choi JK, Lee YH, Kim HM, et al. Transcriptional silencing of the RUNX3 gene by CpG hypermethylation is associated with lung cancer. Biochem Biophys Res Commun. 2004;314:223–8.

42. Melki JR, Vincent PC, Brown RD, Clark SJ. Hypermethylation of E-cadherin in leukemia. Blood. 2000;95:3208–13.

43. Trojan J, Brieger A, Raedle J, Esteller M, Zeuzem S. 5′-CpG island methylation of the LKB1/STK11 promoter and allelic loss at chromosome 19p13.3 in sporadic colorectal cancer. Gut. 2000;47:272–6.

44. Esteller M, Fraga MF, Guo M, Garcia-Foncillas J, Hedenfalk I, Godwin AK. DNA methylation patterns in hereditary human cancers mimic sporadic tumorigenesis. Hum Mol Genet. 2001;10:3001–7.

45. Song SH, Jong HS, Choi HH, Inoue H, Tanabe T, Kim NK, et al. Transcriptional silencing of cyclooxygenase-2 by hypermethylation of the 5′ CpG island in human gastric carcinoma cells. Cancer Res. 2001;61:4628–35.

46. Zöchbauer-Müller S, Fong KM, Maitra A, Lam S, Geradts J, Ashfaq R, et al. 5′ CpG island methylation of the FHIT gene is correlated with loss of gene expression in lung and breast cancer. Cancer Res. 2001;61:3581–5.

47. Ku JL, Yoon KA, Kim IJ, Kim WH, Jang JY, Suh KS, et al. Establishment and characterisation of six human biliary tract cancer cell lines. Br J Cancer. 2002;87:187–93.

48. Suh ER, Ha CS, Rankin EB, Toyota M, Traber PG. DNA methylation down-regulates CDX1 gene expression in colorectal cancer cell lines. J Biol Chem. 2002;277:35759–800.

49. van Engeland M, Roemen GM, Brink M, Pachen MM, Weijenberg MP, de Bruïne AP, et al. K-ras mutations and RASSF1A promoter methylation in colorectal cancer. Oncogene. 2002;21:3792–5.

50. Yang Q, Nakamura M, Nakamura Y, Yoshimura G, Suzuma T, Umemura T, et al. Two-hit inactivation of FHIT by loss of heterozygosity and hypermethylation in breast cancer. Clin Cancer Res. 2002;8:2890–3.

51. Di Vinci A, Infusini E, Nigro S, Monaco R, Giaretti W. Intratumor distribution of 1p deletions in human colorectal adenocarcinoma is commonly homogeneous: indirect evidence of early involvement in colorectal tumorigenesis. Cancer. 1998;83:415–22.

52. Di Vinci A, Infusini E, Peveri C, Sciutto A, Geido E, Risio M, et al. Correlation between 1p deletions and aneusomy in human colorectal adenomas. Int J Cancer. 1998;75:45–50.

53. Tanaka K, Yanoshita R, Konishi M, Oshimura M, Maeda Y, Mori T, et al. Suppression of tumourigenicity in human colon carcinoma cells by introduction of normal chromosome 1p36 region. Oncogene. 1993;8:2253–8.

54. Praml C, Finke LH, Herfarth C, Schlag P, Schwab M, Amler L. Deletion mapping defines different regions in 1p34.2-pter that may harbor genetic information related to human colorectal cancer. Oncogene. 1995;11:1357–62.

55. Ogunbiyi OA, Goodfellow PJ, Gagliardi G, Swanson PE, Birnbaum EH, Fleshman JW, et al. Prognostic value of chromosome 1p allelic loss in colon cancer. Gastroenterology. 1997;113:761–6.

56. Baylin SB, Ohm JE. Epigenetic gene silencing in cancer? A mechanism for early oncogenic pathway addiction? Nat Rev Cancer. 2006;6:107–16.

57. Pradhan S, Bacolla A, Wells RD, Roberts RJ. Recombinant human DNA (cytosine-5) methyltransferase. I. Expression, purification, and comparison of de novo and maintenance methylation. J Biol Chem. 1999;274:33002–10.

58. Bestor TH, The DNA. methyltransferases of mammals. Hum Mol Genet. 2000;92:395–402.

59. Rountree MR, Bachman KE, Herman JG, Baylin SB. DNA methylation, chromatin inheritance, and cancer. Oncogene. 2001;20:3156–65.

60. Fuks F, Burgers WA, Brehm A, Hughes-Davies L, Kouzarides T. DNA methyltransferase Dnmt1 associates with histone deacetylase activity. Nat Genet. 2000;24:88–91.

61. Fuks F. DNA methylation and histone modifications: teaming up to silence genes. Curr Opin Genet Dev. 2005;15:490–538.