Chula Med J Vol. 68 No. 4 October - December 2024 ; 239 - 249 DOI : 10.56808/2673-060X.5490

Original article

H4K20me3 is increased in human bladder cancer tissues and is upregulated by reactive oxygen species in bladder cancer cell lines

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Abstract

Background: Previously, we demonstrated that reactive oxygen species (ROS) triggered oxidative stress, enhanced tumor aggressiveness, and induced a profound change in histone methylation in bladder cancer (BlCa) and reported H4K20me3 upregulation by ROS in hepatocellular carcinoma.
Objective: This study aimed to investigate whether H4K20me3 was upregulated in human BlCa tissues and determine if ROS could induce H4K20me3 formation in BlCa cell lines.
Methods: Immunohistochemical (IHC) staining was performed in 37 BlCa sections and 6 adjacent noncancerous tissues (controls). ROS upregulated H4K20me3 was investigated in three BlCa cell lines: UM-UC-3, VM-CUB-1, and TCCSUP.
Results: The H4K20me3 levels in BlCa tissues increased relative to that in adjacent noncancerous tissues. The IHC score of the H4K20me3 level in BlCa tissues was significantly higher than that in noncancerous controls. H_2O_2 (ROS representative) at 50, 100, and 200 mM significantly induced oxidative stress in UM-UC-3, VM-CUB-1, and TCCSUP cells, respectively, but did not significantly alter cell survival. Western blot and immunofluorescent staining results showed that H_2O_2 treatment markedly increased H4K20me3 formation in all three cell lines.
Conclusion: This study demonstrated that the H4K20me3 levels in BlCa tissues obtained from Thai patients with BlCa increased compared with the levels in adjacent noncancerous tissues. Evidently, ROS upregulated H4K20me3 formation in BlCa cell lines. Perhaps, ROS induced the expression of histone methyltransferases that further caused an increase in H4K20me3 formation. The induction of tumor progression by ROS is well recognized; however, whether ROS induced BlCa progression is mediated by H4K20me3 formation remains to be elucidated.

Keywords: Bladder cancer, H4K20me3, histone methylation, oxidative stress, ROS.

Oxidative stress and epigenetic change are involved in the carcinogenesis and progression of bladder cancer (BlCa). [1] Previously, we demonstrated that hypomethylation of repetitive long interspersed nuclear element-1 (LINE-1) elements was increased in patients with BlCa [2], and reactive oxygen species (ROS) could induce LINE-1 hypomethylation [3] and enhanced LINE-1 protein expression in BlCa cell lines. [4] We showed that ROS not only changed DNA methylation but also caused a profound change in histone methylation in BlCa cells. [5]

Recently, we reported that the H4K20me3 level increased in human hepatocellular carcinoma (HCC) tissues and showed that H4K20me3 upregulation by ROS caused increased aggressiveness of HCC cells. [6] We hypothesized that H4K20me3 levels might be upregulated in human BlCa tissues and that ROS

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Received: May 15, 2024
Revised: June 15, 2024
Accepted: July 8, 2024

could upregulate H4K20me3 formation in BlCa cells similar to that in HCC cells.

H4K20me3 is an inactive chromatin marker abundantly found at heterochromatic regions of repetitive sequences such as satellite repeats and transposable elements. [7] H4K20me3 formation directly contributes to heterochromatin formation and transcriptional repression. [8] H4K20me3 dysregulation is a common event in cancer. In a pioneer study, Fraga MF *et al.* reported that H4K20me3 loss, predominantly at repetitive sequences, is a common hallmark of human cancer cells.[9] H4K20me3 reduction was repeatedly reported in human breast cancer tissues.[10 - 12] In contrast, H4K20me3 formation also increased in human cancers. Benard A, *et al.* demonstrated increased nuclear expression of H4K20me3 in early-stage colon cancer compared with paired normal samples.[13] Zhou M *et al.* also reported that the H4K20me3 level increased in esophageal squamous cell carcinoma, and such elevation was associated with poor prognosis.[14] Hitherto, only one study investigated the H4K20me3 level in human BlCa tissues. Schneider AC *et al.* performed immunohistochemical (IHC) staining on microarray tissue samples from patients with BlCa and showed that the H4K20me3 levels in BlCa tissues were lower than those in normal urothelium. [15] Therefore, more studies of IHC staining of H4K20me3 in urinary bladder tissues of patients with BlCa are needed.

In this study, we aimed to investigate the H4K20me3 level in BlCa tissues by IHC and explore whether H4K20me3 formation was increased in BlCa cell lines under oxidative stress conditions.

Materials and methods

IHC staining

IHC staining for H4K20me3 was performed in paraffin-embedded bladder sections obtained from patients with BlCa. [4, 5] The research protocol was approved by the Ethics Committee of the Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand (IRB no. 163/64).

Briefly, sections were deparaffinized and rehydrated. Antigens were retrieved by boiling in sodium citrate buffer (pH 6.0) for 15 min. Endogenous peroxidase was inactivated by incubating with 0.3% H_2O_2 for 30 min, and nonspecific binding was blocked by incubating with 10.0% normal horse serum for 1 h. Sections were further incubated with the H4K20me3 antibody (1:200, ab5093, Abcam) at 4°C overnight. After washing, sections were incubated with the biotinylated universal antibody for 1 h, followed by the ABC reagent for 30 min (Vectastain Elite ABC Universal kit, PK7200). The color of the immunocomplexes was developed by immersing sections in 0.2% diaminobenzidine solution containing 0.005% H_2O_2. After counterstaining with hematoxylin, sections were dehydrated and mounted.

Relative levels of H4K20me3 were evaluated based on the percentage of positive cells (graded into 0, 0.0%; 1, 1.0% - 25.0%; 2, 26.0% - 50.0%; 3, 51.0% - 75.0%; and 4, 75.0% 100.0%) and intensity of staining (0 = negative, 1 = +, 2 = ++, 3 = +++, 4 = ++++), averaged from five microscopic high-power fields. The IHC score was calculated from a score of % positive cells (0 - 4) multiplied by the score of intensity (0 - 4) as described previously. [6] Representative micrographs of H4K20me3 staining for IHC scores of 0, 4, 8, 12, and 16 are shown in **Figure 1.**

Cell culture condition

BlCa cell lines including UM-UC-3, VM-CUB-1, and TCCSUP cells (ATCC, VA, USA) were maintained in Dulbecco's medium supplemented with 10.0% fetal bovine serum (HyClone Laboratories, Logan, UT), 1.0% penicillin–streptomycin (HyClone Laboratories) under 37°C, 5.0% CO_2, and 100.0% humidity. H_2O_2 was used as an ROS representative to induce oxidative stress in BlCa cells. The optimal concentrations of H_2O_2 that could induce oxidative stress in BlCa cells but did not significantly reduce cell survival were 50 μM for UM-UC-3, 100 μM for VM-CUB-1, and 200 μM for TCCSUP.

Intracellular ROS production

Intracellular ROS production was determined by the dichlorodihydrofluorescein diacetate (DCFH-DA) assay. [5] BlCa cells (10,000 cells/well) were seeded in 96- black well plate and incubated at 37°C and 5.0% CO_2 overnight. After the medium was removed, cells were incubated with the freshly prepared DCFH-DA solution (0.5 mm in a serum-free medium) for 30 min at 37°C. After washing with phosphate-buffered saline (PBS), cells were treated with a medium containing varied concentrations of H_2O_2. The fluorescent intensities at 480 nm for excitation and 535 nm for emission were measured at the beginning (0) and at 1, 6, and 24 h after H_2O_2 treatment. The arbitrary

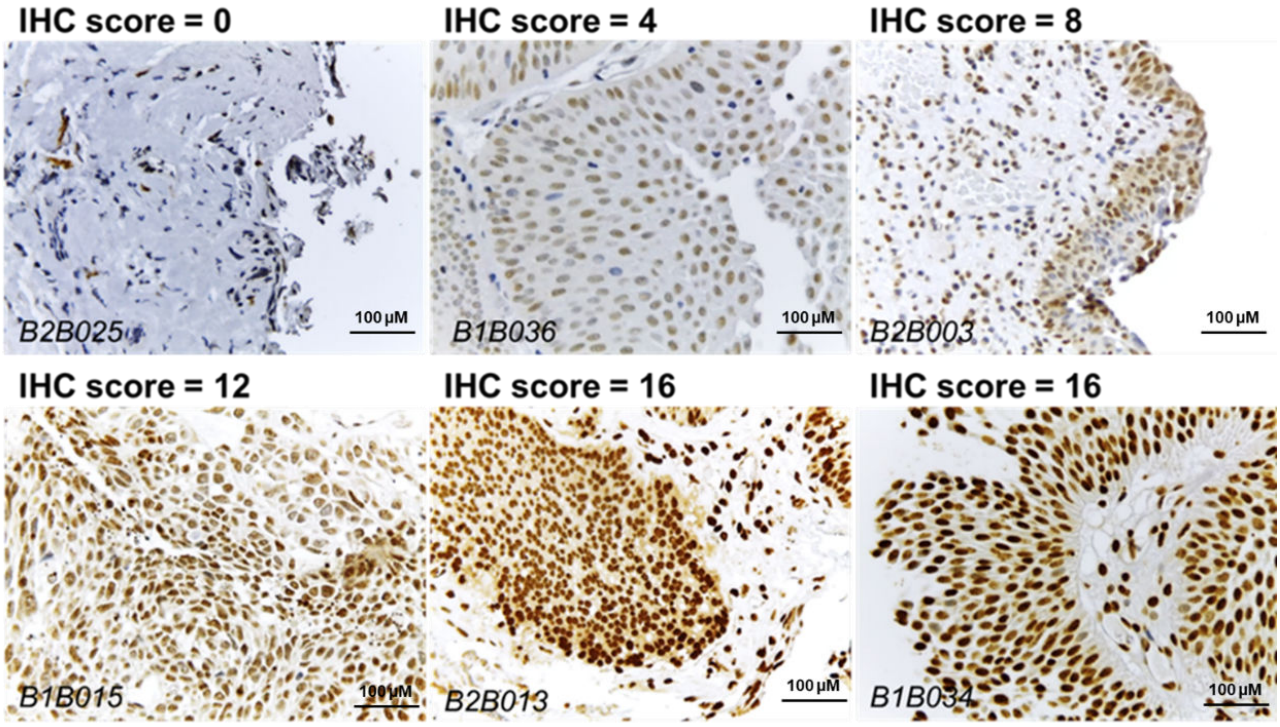

Figure 1. Representative micrographs of H4K20me3 staining for IHC scores of 0, 4, 8, 12 and 16. The higher IHC score indicates the stronger positivity of H4K20me3 staining.

fluorescent unit indicated that the intracellular ROS level was calculated from the ratio of the fluorescent intensity at each time point to the fluorescent intensity at the beginning (0 h).

Protein oxidation measurement

Protein carbonylation was measured as an indicator of protein oxidation by ROS. After H_2O_2 treatment, total protein was extracted from cells using a radioimmunoprecipitation assay buffer containing a protease inhibitor cocktail. Protein concentration was determined by the bicinchoninic acid method. Protein carbonyl was measured as described previously. [5] Briefly, cell lysates were incubated with 10 mM 2, 4-dinitrophenylhydrazine (DNPH, as a test) or 2 N HCl (as a reagent blank) for 60 min in the dark. Then, cold 20.0% trichloroacetic acid (TCA) was added and incubated on ice for 15 min. Yellow pellets were collected by centrifugation at 10,000 xg, 4°C for 15 min and washed with ethanol: ethyl acetate (1 : 1). The washed pellets were further redissolved in 6 M guanidine HCl at 60°C for 30 min. The absorbance at 375 nm (A375) was measured. The protein carbonyl level (nmol/mg of protein) was calculated from the following formula: $((A375_{DNPH} - A375_{HCl}) \times 45.5)$/ protein concentration. [16]

RNA extraction and quantitative reverse-transcription polymerase chain reaction (RT-PCR)

Total RNA was extracted from BlCa cells using the GF-1 total RNA extraction kit (Vivantis, Buckinghamshire, Malaysia). The concentration of total RNA was measured using the NanoDrop spectrophotometer (Thermo Fisher Scientific, MA, USA). cDNA was synthesized from 1 µg of the RNA template using the reverse-transcription kit (Thermo Fisher Scientific). qPCR was performed using SYBR Green PCR master mix (Biotechrabbit, Germany) and specific primers for Nrf2 (F: 5'-ACACGGTCCA CAGCTCATC-3', R: 5'-TGCCTCCAAGTATGT CAATA-3'), NQO1 (F: 5'-GAAGAGCACTGAT CGTACTGGC-3' and R: 5'-GGATACTGAAAGT TCGCAGGG-3'), and GAPDH (F: 5'- CAAGGTCA CCATGACAACTTTG-3', R: 5'-GTCCACCACCC TGTTGCTGTAG-3').[6] PCR amplification was performed in the QuantStudio™ 6 RealTime PCR system (Thermo Fisher Scientific). The relative mRNA expression was calculated by the $2^{-\triangle\triangle CT}$ method, which was normalized against GAPDH as the internal control.

Histone protein extraction

The cell lysate was resuspended in a cold hypotonic lysis buffer containing a protease inhibitor cocktail, phenylmethylsulfonyl fluoride, and dithiothreitol and rotated at 4°C for 1 h. Intact nuclei pellets were collected by centrifugation and resuspended in H_2SO_4 by rotating at 4°C overnight. After centrifugation, the supernatant was transferred to a new microcentrifuge tube. Histone proteins were precipitated by incubating in ice-cold 100.0% TCA for 30 min. Histone pellets were carefully collected and washed with 100.0% acetone. Finally, histone pellets were dried at room temperature and redissolved in ultrapure water.

Western blot analysis

Histone proteins were separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (12.0% gel, running at 100 V for 20 min, then 150 V for 1 h). After electrophoresis, proteins were transferred to a PVDF membrane using a wet tank blotting system (180 mA for 60 min). The membrane was incubated with 5.0% skim milk for 1 h and incubated with H4K20me3 antibody (3 µg, ab5093, Abcam) at 4°C overnight. After washing, the membrane was incubated with an anti-rabbit IgG HRP-linked secondary antibody (#7074, Cell Signaling) for 1 h. Histone H4 was detected as the loading control using 3 µg of histone H4 antibody (ab10158, Abcam, Cambridge, UK). The immunocomplex signal was developed by the SuperSignal West Femto MaximuM Sensitivity Substrate (Thermo Fisher Scientific) and imaged using the ChemiDoc™ Touch imaging system (Bio-Rad Laboratories, CA, USA).

Immunofluorescent staining for H4K20me3

BlCa cells were grown on coverslips. After H_2O_2 treatment, cells were fixed with 4.0% paraformaldehyde in PBS for 10 min, permeabilized with 0.5% triton X-100 for 10 min, and blocked by incubating with normal horse serum at room temperature for 1 h. Cells were incubated with the H4K20me3 antibody (ab5093, Abcam) at 4°C overnight. After washing, cells were incubated with Alexa Fluor®488 goat anti-rabbit IgG H&L (Thermo Fisher Scientific) for 1 h in the dark. The nuclei were stained by DAPI (Sigma-Aldrich) for 10 min. The stained coverslips were mounted with the Fluoromount-G™ Mounting Medium (Invitrogen by Thermo Fisher Scientific).

Statistical analysis

Data were presented as means ± standard deviations (SD) or standard error of the mean (SEM), as appropriate. Categorical data were presented as frequencies and percentages. Student's, *t* - test or Mann-Whitney test was used for the comparison of variables between the two independent groups. GraphPad Prism 10 (GraphPad, La Jolla, CA) was employed for graphs and statistical analyses. $P < 0.05$ was considered significant.

Results

Increased H4K20me3 level in BlCa tissues compared with adjacent noncancerous tissues

BlCa tissues were obtained from 37 patients with BlCa. The demographic and clinical characteristics of these patients are shown in **Table 1.** Ideally, normal urinary bladder tissue sections should be used as normal controls for comparing H4K20me3 levels between normal urothelial and BlCa cells. In this study, we did not have normal urothelial tissue samples. However, some BlCa tissue samples (n = 6) contained noncancerous areas located near the cancerous tissues, so these adjacent noncancerous tissues were used as controls. To identify cancerous and noncancerous areas, hematoxylin and eosin staining was performed in all sections **(Supplementary Figure 1).**

The IHC result revealed that the H4K20me3 level in BlCa tissues was markedly increased compared with that in adjacent noncancerous controls **(Figure 2A).** Furthermore, both the papillary bladder tumor (nonmuscle-invasive, **Figure 2B**) and muscle-invasive bladder tumor **(Figure 2C)** tissues exhibited higher H4K20me3 levels than the noncancerous bladder tissues **(Figure 2A).** Based on our quantitative IHC score, the IHC score of the H4K20me3 level in BlCa tissues was significantly higher than that in noncancerous tissues **(Figure 2D).** H4K20me3 staining for all sections is shown in **Supplementary Figure 2.**

Table 1. The characteristics of the studied BlCa patients.

Characteristics	Frequency (%)
N of patients	37
Age (mean ± SD) (years)	70.6 ± 14.0
Gender	
Male	32 (86.5)
Female	5 (13.5)
Recurrence	
Yes	25 (67.6)
No	12 (32.4)
Tumor type	
Papillary/non muscle invasive	27 (73.0)
Muscle invasive	10 (27.0)
Tumor grading (n = 32)	
Low-grade	13 (40.6)
High-grade	19 (59.4)
Surgical approach (n = 36)	
TUR-BT*	33 (91.7)
Radical cystectomy	3 (8.3)

*TUR-BT, Transurethral resection of bladder tumor

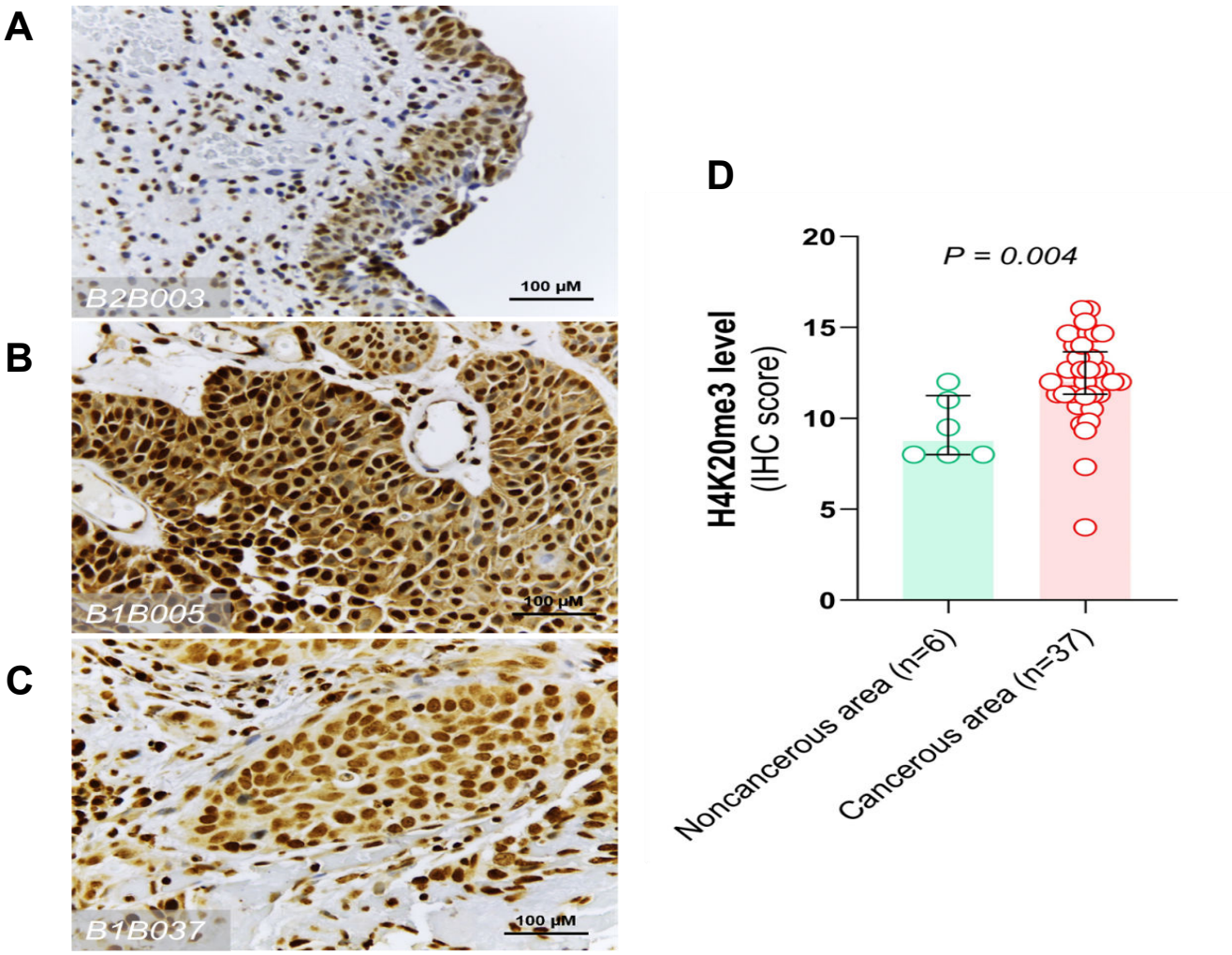

Figure 2. Representation micrographs of H4K20me3 levels compared between BlCa tissues and adjacent noncancerous tissues; **(A)** adjacent noncancerous bladder tissue; **(B)** papillary bladder tumor tissue; **(C)** muscle-invasive bladder tumor tissue; **(D)** quantitation of H4K20me3 formation using IHC score revealed that H4K20me3 level in noncancerous tissues was significantly lower than in BlCa tissues. Magnification: 400x. Bars and error bars indicate medians and interquartile ranges, respectively.

Sublethal concentration of H_2O_2 induced oxidative stress in BlCa cell lines

H_2O_2 is the most common representative of ROS used in in vitro experiments for the induction of oxidative stress.[17] High H_2O_2 concentrations inevitably induce apoptosis. [18] To investigate the effect of oxidative stress on H4K20me3 upregulation in BlCa cells, we sought to determine the H_2O_2 concentration that could provoke cellular oxidative stress but not significantly alter cell survival. Based on our previous cytotoxicity data from the MTT assay, we used the sublethal concentrations of H_2O_2 at 50, 100, and 200 mM to induce oxidative stress in UM-UC-3, VM-CUB-1, and TCCSUP, respectively.

The induction of oxidative stress in UM-UC-3, VM-CUB-1, and TCCSUP cells after H_2O_2 treatment was investigated. Intracellular ROS production after H_2O_2 exposure for 1, 6, and 24 h was measured using the DCHF-DA method. Intracellular ROS generation was increased by exposure time in all BlCa cell lines. After 24 h of exposure, ROS levels were significantly increased in VM-CUB-1 and TCCSUP cells **(Figure 3).**

The protein carbonyl content as an indicator of protein oxidation was measured. The results clearly demonstrated that the levels of protein carbonyl content after H_2O_2 treatment were significantly increased in all three BlCa cell lines compared with untreated controls **(Figure 4).** The Nrf2–ARE signaling pathway is the main cellular response against oxidative stress. [19] In this study, expressions of Nrf2 and NQO1 mRNA in BlCa cells treated with H_2O_2 for 6 and 24 h were investigated. The results revealed that the expression levels of both Nrf2 and NQO1 mRNA were significantly increased in all three BlCa cells after H_2O_2 treatment **(Figure 5).** This finding indicated that BlCa cells treated with H_2O_2 were undergoing oxidative stress.

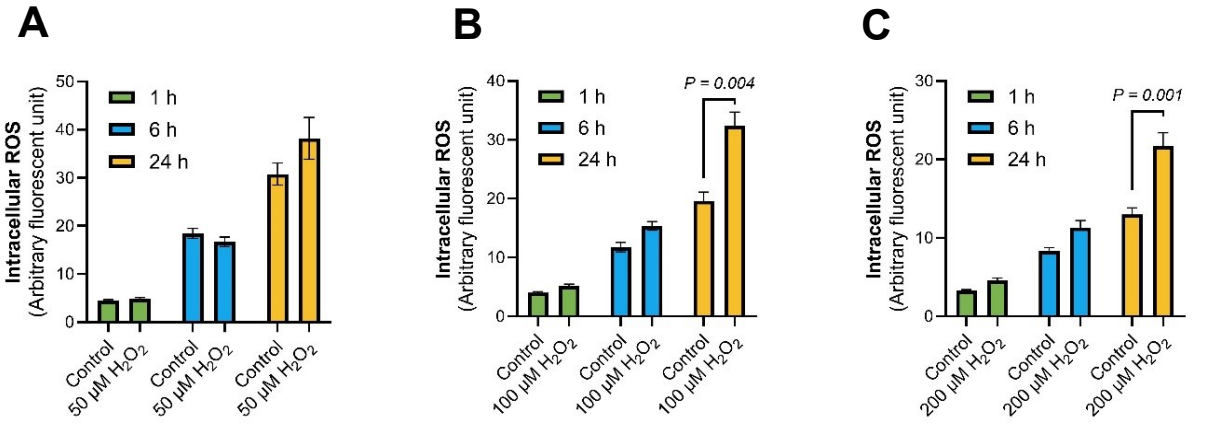

Figure 3. Intracellular ROS production in BlCa cells after exposure to H_2O_2 for 1, 6, and 24 h. **(A)** UM-UC-3 cells; **(B)** VM-CUB-1 cells; **(C)** TCCSUP cells. Bars and error bars indicate mean ± SEM.

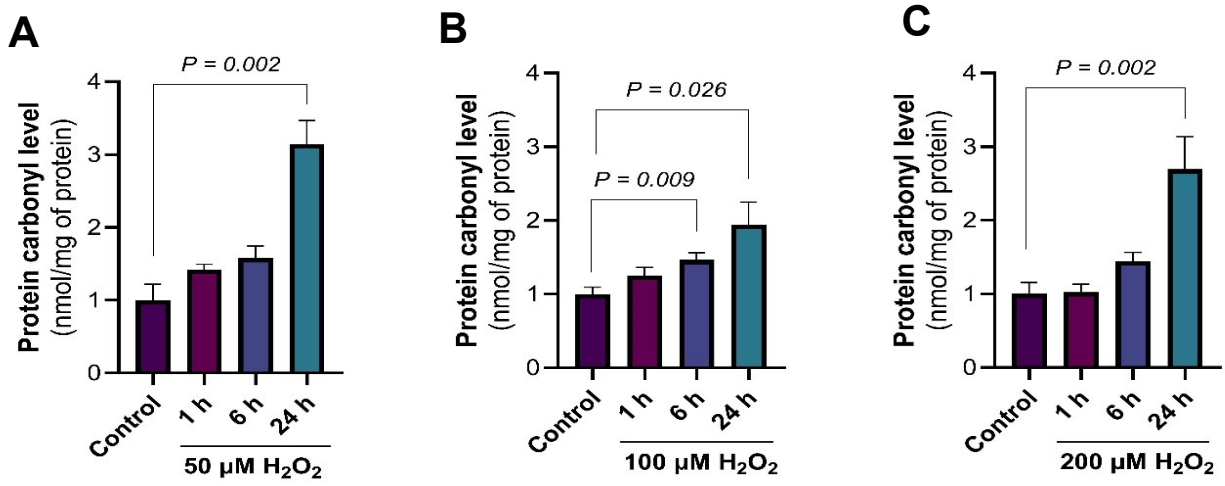

Figure 4. Protein carbonyl content in BlCa cells treated with H_2O_2 for 1, 6, and 24 h. **(A)** UM-UC-3 cells; **(B)** VM-CUB-1 cells; **(C)** TCCSUP cells. Bars and error bars indicate mean ± SEM.

H4K20me3 was upregulated by H_2O_2 in BlCa cells

Oxidative stress can alter DNA and histone methylation. [20] Therefore, we further explored whether ROS could increase H4K20me3 formation in BlCa cells. UM-UC-3, VM-CUB-1, and TCCSUP cells were treated with H_2O_2 for 6 and 24 h. H4K20me3 was detected and quantified by Western blot and immunodetection. The result showed that the H4K20me3 level in H_2O_2-treated cells time-dependently increased **(Figure 6A)**. The result of band quantitation by a densitometer revealed that the H4K20me3 levels in BlCa cells after H_2O_2 treatment for 6 and 24 h were significantly increased compared with that in untreated control cells, particularly in UM-UC-3 and VM-CUB-1 cells **(Figure 6B)**.

H4K20me3 upregulation by H_2O_2 in BlCa cells was confirmed by immunofluorescent staining. The result showed that the fluorescent signal of H4K20me3 was noticeably increased in H_2O_2-treated cells compared with that of untreated controls **(Figure 7)**.

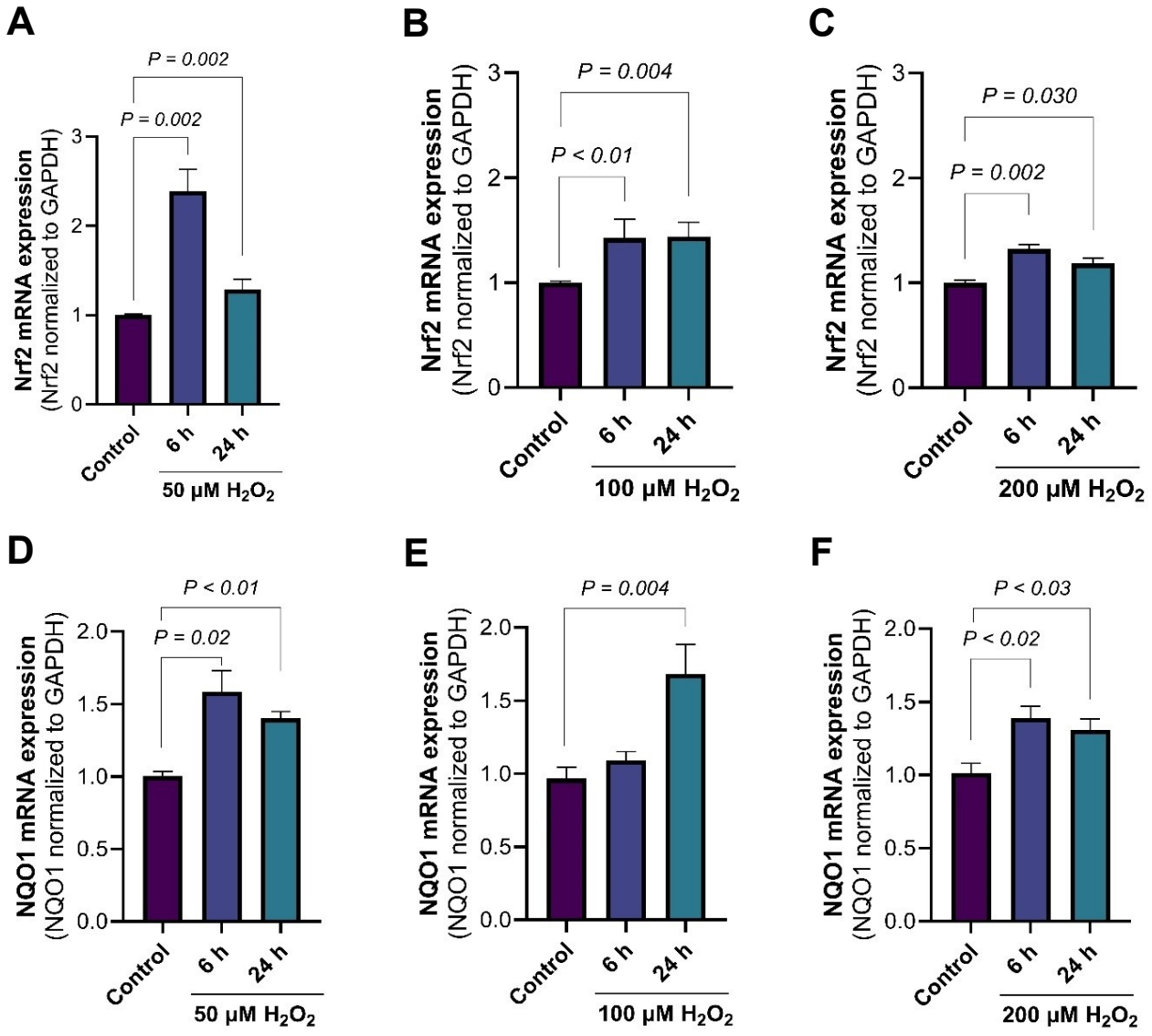

Figure 5. Transcript expression of Nrf2 and NQO1 in BlCa cells treated with H_2O_2 for 6 and 24 h; **(A and D)** UM-UC-3 cells; **(B and E)** VM-CUB-1 cells; **(C and F)** TCCSUP cells; Bars and error bars indicate mean ± SEM.

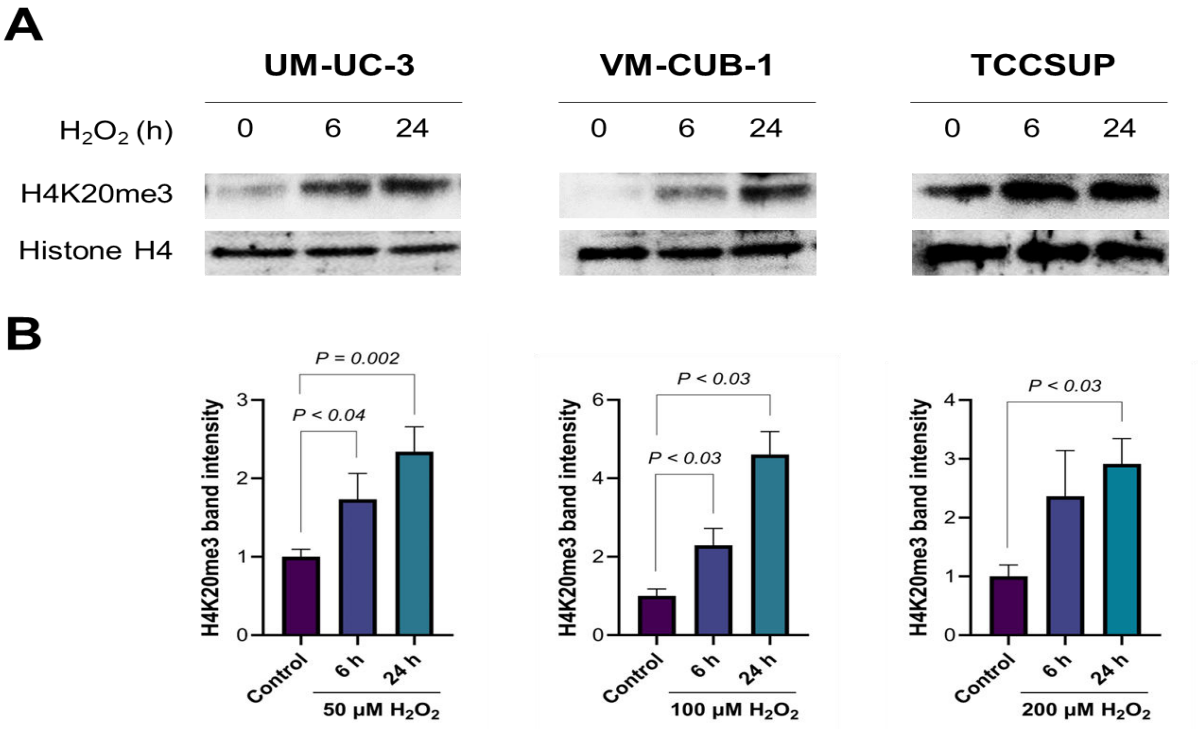

Figure 6. Western blot analysis showing upregulation of H4K20me3 in BlCa cells (UM-UC-3, VM-CUB-1 and TCCSUP) following H_2O_2 treatment; **(A)** Representative western blot bands of H4K20me3 and histone H4 (loading control); **(B)** Quantitative comparison of H4K20me3 band intensities. Bars and error bars indicate mean ± SEM. The full uncropped SDS-PAGE gel is shown in the Supplementary **Figure 3.**

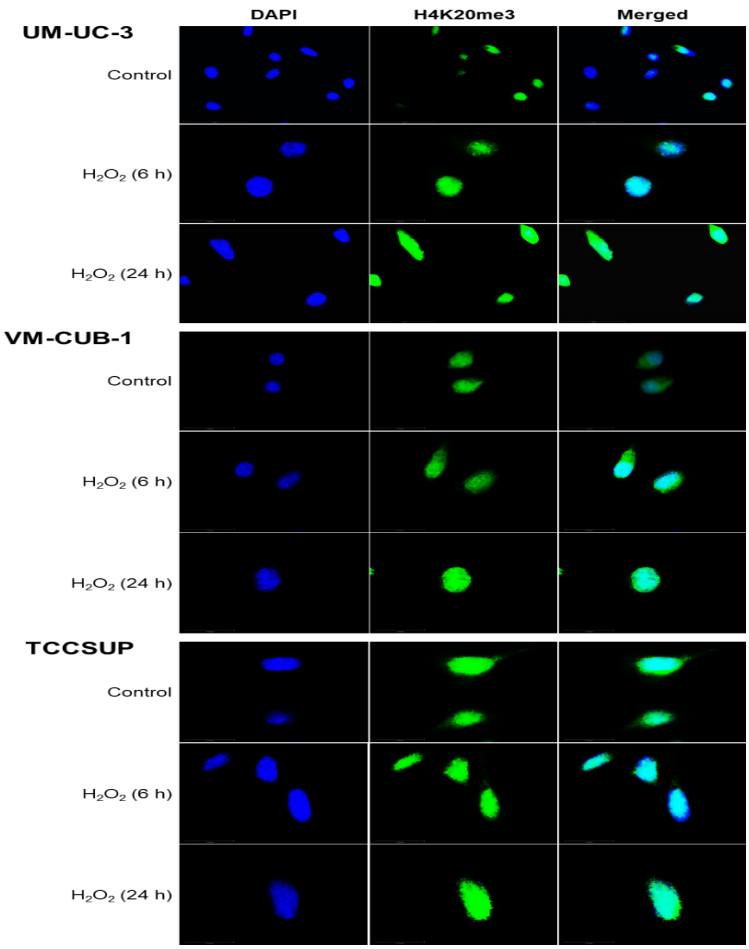

Figure 7. Immunofluorescent staining of H4K20me3 in BlCa cells treated with H_2O_2 for 6 and 24 h. H4K20me3 level was clearly increased following H_2O_2 treatment in all three cell lines, **(A)** UM-UC-3, **(B)** VM-CUB-1, and **(C)** TCCSUP. Magnification: 1000x.

Discussion

H4K20me3, a well-known chromatin mark, triggers the formation of constitutive heterochromatin and is found mainly at pericentromeres and telomeres to inactivate gene-poor and repetitive elements. [8] Altered H4K20me3 levels have been reported in many cancers, and H4K20me3 loss in repetitive sequences is suggested to be a common hallmark of human tumor cells. [9] However, evidence of H4K20me3 formation in BlCa is still limited. To our knowledge, only one study in 2011 reported a reduction of H4K20me3 level in BlCa tissues compared with normal urothelial tissues. [15] Thus far, no histological investigation of H4K20me3 in Thai patients with BlCa has been performed. Therefore, this is the first study that immunohistochemically investigated H4K20me3 formation in BlCa tissues obtained from Thai patients. We demonstrated that the H4K20me3 level in BlCa tissues was significantly higher than that in adjacent noncancerous bladder tissues. Our finding did not corroborate the results of Schneider AC, *et al.* [15] who showed that H4K20me3 levels were decreasing from the normal urothelium over nonmuscle-invasive BlCa and muscle-invasive BlCa to metastatic BlCa. [15] However, they found that high H4K20me3 levels were associated with poor prognosis in patients with muscle-invasive BlCa, as it increased the risk of BlCa-specific mortality. [15] In addition to our finding of an increased H4K20me3 level in cancerous tissues, increased H4K20me3 levels were also found in colorectal cancer (TMN stages I - III) [13] and esophageal squamous cell carcinoma. [14] Furthermore, the high H4K20me3 level was associated with poor prognosis in esophagus cancer [14] and muscle-invasive BlCa. [15] In our recent study in HCC, the inhibition of H4K20me3 formation by A-196, a selective inhibitor of SUV420h1 and SUV420h2, could reduce tumor aggressivity in HCC cells. [6] These findings suggest that increased H4K20me3 level promotes tumor progression and aggressivity. Thus, H4K20me3 might have a critical role in BlCa progression. The oncogenic function of H4K20me3 in BlCa progression needs to be warranted in further study.

Factors or conditions that upregulate H4K20me3 formation in cancer cells are not fully known. UVA radiation increases the H4K20me3 level in HAP1 cells, a human cell line derived from KBM-7 leukemic cell line, and H4K20me3 takes on a vital role in nonhomologous end-joining repairing process. [21] Oxidative stress can also induce changes in DNA and histone methylation. [5, 20] We reported that the H4K20me3 level was increased in human HCC tissues, and H_2O_2 can upregulate H4K20me3 in HCC cell lines. [6] In addition, H_2O_2 could promote cell migration and invasion in HCC [6, 22] and BlCa cells. [4] In this study, we demonstrated that H_2O_2 could increase H4K20me3 levels in BlCa cell lines. Kloypan C, *et al*. investigated whether DNA hypomethylation of long LINE-1 induced by ROS was mediated through the depletion of the methyl donor S-adenosylmethionine (SAM). [23] They found that ROS depletion induced glutathione synthesis in BlCa cells through the transsulfuration pathway, leading to homocysteine reduction, which consequently caused the diminution of SAM and LINE-1 hypomethylation. [23] Phoyen S. *et al.* demonstrated that H_2O_2 triggered oxidative stress and induced H4K20me3 formation in HepG2 and Huh7 cells together with the upregulation of the mRNA expression of histone methyltransferase Suv420h2 (enzyme responsible for H4K20me3 formation). [6] According to the result of the present study, ROS was assumed to induce the expression of histone methyltransferases (Suv420h2) that further caused increased H4K20me3 formation. Further experimental studies should be conducted to warrant this speculation. Because increased oxidative stress is well documented in BlCa [2], we speculate that oxidative stress upregulates H4K20me3 formation in BlCa cells that further change the pattern of gene expression to facilitate tumor progression. However, this speculation must be experimentally verified in further studies.

This study has some limitations. The sample size for H4K20me3 IHC staining was still small. Thus, the current finding must be validated in a larger population. Whether the H4K20me3 upregulation by H_2O_2 could enhance BlCa progression was not examined. The expression of histone methyltransferase (SUV420h2) responsible for H4K20me3 formation was not investigated, which would have revealed the mechanistic connection to increased H4K20me3 levels. Although H_2O_2 is the most common inducer of oxidative stress in many studies, it is also interesting to explore whether other oxidative stress inducers can upregulate H4K20me3 formation in BlCa cells similarly to H_2O_2.

Conclusion

In this study, we immunohistochemically demonstrated that H4K20me3 levels were increased in human BlCa tissues compared with those in adjacent noncancerous tissues. In addition, ROS could induce H4K20me3 formation in BlCa cell lines. However, whether an increased H4K20me3 formation by ROS contributes to BlCa progression remains to be elucidated.

Acknowledgements

This project was also funded by National Research Council of Thailand (NRCT) and Chulalongkorn University (N42A650265 to C.B.), and the Office of national higher education science research and innovation policy council by the Program management unit for Competitiveness (PMUC) (C10F640117 to C.B.). S.K. received a scholarship for thesis support from Graduate School, Chulalongkorn University. S.P. was awarded the Thailand Research Fund under the Royal Golden Jubilee Ph.D. Scholarship (PHD/0030/2558, 4.O.CU/58/A.1).

Conflicts of interest statement

All authors have completed and submitted the International Committee of Medical Journal Editors Uniform Disclosure Form for Potential Conflicts of Interest. None of the authors disclose any conflict of interest.

Data sharing statement

All data generated or analyzed during the present study are included in this published article. Further details are available for noncommercial purposes from the corresponding author on reasonable request.

Supplementary material

Supplementary data to this article can be found online at file:///C:/Users/User/Downloads/Supplementary+Figures%20(1).pdf

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