Acknowledgments The authors thank Mrs. Shigeko Suzuki for her technical assistance in preparing the histological specimens. This work was supported by Health and Labour Sciences Research Grants (Research on Food Safety) from the Ministry of Health, Labour and Welfare of Japan.

Conflict of interest The authors disclose that there are no competing financial interests that could inappropriately influence the outcome of this study.

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Toxicology Letters 219 (2013) 203–210

Contents lists available at SciVerse ScienceDirect

Toxicology Letters

journal homepage: www.elsevier.com/locate/toxlet

Aberrant activation of M phase proteins by cell proliferation-evoking carcinogens after 28-day administration in rats

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HIGHLIGHTS

- This study aimed to identify early prediction markers of carcinogens in rats.
- Cellular distribution of cell cycle proteins was analyzed after 28-day treatment.
- Cell proliferation-evoking carcinogens induced activation of M phase proteins.
- Carcinogens lacking proliferative activity did not have these effects.
- Cell proliferation and M phase proteins might functions as an early prediction unit.

ARTICLE INFO

Article history:
Received 19 January 2013
Received in revised form 12 March 2013
Accepted 15 March 2013
Available online 25 March 2013

Keywords:
Carcinogen
M phase
Cell proliferation
Immunohistochemical analysis

ABSTRACT

We have previously reported that hepatocarcinogens increase liver cells expressing $p21^{Cip1}$, a G_1 checkpoint protein and M phase proteins after 28-day treatment in rats. This study aimed to identify early prediction markers of carcinogens available in many target organs after 28-day treatment in rats. Immunohistochemical analysis was performed on Ki-67, $p21^{Cip1}$ and M phase proteins [nuclear Cdc2, phospho-Histone H3 (p-Histone H3), Aurora B and heterochromatin protein 1α (HP1α)] with carcinogens targeting different organs. Carcinogens targeting thyroid (sulfadimethoxine; SDM), urinary bladder (phenylethyl isothiocyanate), forestomach (butylated hydroxyanisole; BHA), glandular stomach (catechol; CC), and colon (2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine and chenodeoxycholic acid) were examined using a non-carcinogenic toxicant (caprolactam) and carcinogens targeting other organs as negative controls. All carcinogens increased Ki-67$^+$, nuclear Cdc2$^+$, p-Histone H3$^+$ or Aurora B$^+$ carcinogenic target cells, except for both colon carcinogens, which did not increase cell proliferation. On the other hand, $p21^{Cip1+}$ cells increased with SDM and CC. HP1α responded only to BHA. Results revealed carcinogens evoking cell proliferation concurrently induced cell cycle arrest at M phase or showing chromosomal instability reflecting aberration in cell cycle regulation, irrespective of target organs, after 28-day treatment. Therefore, M phase proteins may be early prediction markers of carcinogens evoking cell proliferation in many target organs.

1. Introduction

In general, the method for evaluating carcinogenicity is a bioassay in which rodents are treated with a chemical for their entire 1.5- or 2-year lifespan. Carcinogenicity studies using experimental animals are time-consuming, expensive, and use many animals. However, there is no commonly rapid means for evaluating the carcinogenic potential of chemicals. Alternative animal models using medium-term carcinogenesis models (Tamano, 2010) or genetically modified animals using transgenic or gene-targeting technologies (Eastin, 1998) are also expensive and time-consuming or have limited target organs. Toxicogenomic approaches for the prediction of carcinogenic potential in each target organ appear promising. However, they are also expensive and require some integrative methodologies between different laboratories sharing an expression database (Uehara et al., 2011).

Development of nuclear enlargement is sometimes observed in carcinogenic target cells after repeated administration of

Abbreviations: SDM, sulfadimethoxine; PEITC, phenylethyl isothiocyanate; BHA, butylated hydroxyanisole; CC, catechol; PhIP, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine; CDCA, chenodeoxycholic acid; p-Histone H3, phospho-Histone H3; HP1α, heterochromatin protein 1α; CL, caprolactam.

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http://dx.doi.org/10.1016/j.toxlet.2013.03.012

carcinogens, irrespective of genotoxic potential, from the early stages of exposure in experimental animals (Adler et al., 2009; Allen et al., 2004). This nuclear enlargement is typically observed in the liver and kidney. It is often termed cytomegaly in cases of liver cells characterized by the presence of hepatocytes that are enlarged because of increased cytoplasmic volume, and karyomegaly when it occurs in renal tubular cells. Recent studies have shown that ochratoxin A, a representative renal carcinogen that can typically induce karyomegaly, induces aberrant expression of cell cycle-related proteins in the proximal tubular areas of the outer stripe of the outer medulla with karyomegaly (Adler et al., 2009). Generation of karyomegaly/cytomegaly suggests cell cycle aberration causing chromosomal instability through nuclear division during mitosis. Aberrant mitosis, such as chromosomal missegregation and cytokinesis failure occurring as a result of checkpoint dysfunction of the cell cycle, can induce tetraploidy/aneuploidy (Ichijima et al., 2010). This suggests that this aberrant expression of cell cycle-related proteins may eventually cause carcinogenicity in association with the development of chromosomal instability. Therefore, we hypothesize that an early event, which disrupts cell cycle regulation, triggers the carcinogenic response in the molecular mechanism responsible for the development of cytomegaly/karyomegaly.

We have previously analyzed cell cycle-related proteins in a 28-day study of repeated hepatocarcinogen administration to induce cytomegaly in rats (Yafune et al., 2013). These responses suggested hepatocarcinogens, irrespective of cytomegaly-inducing potential, induced an increase in the liver cell population immunoreactive for $p21^{Cip1}$ and Aurora B, suggestive of those undergoing G_1 arrest and chromosomal instability, respectively. We also found that hepatocarcinogens that evoke cell proliferation might cause M phase arrest of liver cells, judging from increased cell population expressing nuclear Cdc2, phospho-Histone H3 (p-Histone H3), and heterochromatin protein 1α (HP1α), accompanied with apoptosis. The obtained results suggested that a combination of these cell cycle proteins might be an early prediction battery of markers of hepatocarcinogens in a 28-day treatment scheme in rats.

There is a need for an available prediction tool to assess the carcinogenic potential of chemicals. To establish a short-term carcinogenicity screening system, it is reasonable to focus on common cellular responses in specific target organs. In the present study, based on our previous results on hepatocarcinogens, expression of these candidate proteins was explored in other target organs, including the thyroid, urinary bladder, forestomach, glandular stomach and colon, after 28-day treatment with organ-specific carcinogens in rats.

2. Materials and methods

2.1. Chemicals

Butylated hydroxyanisole (BHA; CAS No. 25013-16-5, ≥98.0%), caprolactam (CL; CAS No. 105-60-2, 98%), and catechol (CC; CAS No. 120-80-9, >99.0%) were purchased from Wako Pure Chemicals Industries, Ltd. (Osaka, Japan). Chenodeoxycholic acid (CDCA; CAS No. 474-25-9, ≥98.0%) and phenylethyl isothiocyanate (PEITC; CAS No. 2257-09-2, ≥97.0%) were obtained from Tokyo Chemical Industry Corporation (Tokyo, Japan). Sulfadimethoxine sodium salt (SDM; CAS No. 122-11-2) was obtained from Sigma–Aldrich Corporation (St. Louis, MO, USA). 2-Amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP; CAS No. 105650-23-5, ≥98.0%) was obtained from Nard Institute (Hyogo, Japan).

2.2. Animal experiments

Five-week-old male F344/NSlc rats were purchased from Japan SLC, Inc. (Shizuoka, Japan) and acclimatized to a powdered basal diet (CRF-1 diet; Oriental Yeast Co., Tokyo, Japan) and tap water *ad libitum*. They were housed in stainless steel cages in a barrier-maintained animal room on a 12 h light–dark cycle and conditioned at 23 ± 3 °C with relative humidity of 50 ± 20%. After a 1-week acclimatization period, animals were randomized into groups of 10 each and treated with carcinogens or non-carcinogens for 28 days.

Animals were treated with carcinogenic doses of carcinogens targeting either the thyroid, urinary bladder, forestomach, glandular stomach or colon for 28 days. Groups received either SDM (1000 ppm in drinking water) targeting the thyroid, PEITC (1000 ppm in diet) targeting the urinary bladder, BHA (20,000 ppm in diet) targeting the forestomach, CC (8000 ppm in diet) targeting the glandular stomach, or CDCA (1000 ppm in diet) or PhIP (400 ppm in diet) targeting the colon. The dose of SDM and CDCA has been shown to promote carcinogenesis in the thyroid and colon, respectively, in rats (Ghia et al., 1996; Imai et al., 2004). With regard to PEITC, BHA, CC and PhIP, the dose has been shown to induce tumors in each target organ (Hagiwara et al., 2001; Ito et al., 1991; Kaneko et al., 2002; Sugiura et al., 2003). CL (10,000 ppm in diet) was selected as a non-carcinogenic control compound, exhibiting positivity in some genotoxicity studies (IARC, 1999). This compound has shown no carcinogenic effect in any organs with ≥7500 ppm in diet (Fukushima et al., 1991; NTP, 1982). Untreated control animals were given basal diet and tap water *ad libitum* for 28 days.

One day after the 28-day treatment, all animals were sacrificed by exsanguination from the abdominal aorta under deep anesthesia using CO_2/O_2, and target organs were removed. Target organs were fixed with 4% paraformaldehyde in 0.1 M sodium phosphate buffer solution (pH 7.4; Wako Pure Chemicals Industries, Ltd.). At necropsy, the urinary bladder was inflated by transurethral instillation of a paraformaldehyde solution, and the stomach and colon were instilled with a paraformaldehyde solution to facilitate mucosal fixation. The following samples were taken from fixed tissues and prepared for paraffin embedding: bilateral lobes of the thyroid; two longitudinal slices of the urinary bladder; three longitudinal slices of the stomach including forestomach and glandular stomach; and three cross cut pieces each from proximal, medial, and distal portions of the colon.

All procedures in this study were conducted in compliance with the Guidelines for Proper Conduct of Animal Experiments (Science Council of Japan, June 1, 2006) and according to the protocol approved by the Animal Care and Use Committee of the Tokyo University of Agriculture and Technology.

2.3. Histopathology and immunohistochemistry

Three micrometer sections of paraffin-embedded tissues from the thyroid, urinary bladder, stomach, and colon were stained with hematoxylin and eosin for histopathological examination and subjected to immunohistochemistry.

Immunohistochemistry was performed using the Vectastain® Elite ABC Kit (Vector Laboratories Inc., Burlingame, CA, USA) with 3,3′-diaminobenzidine/H_2O_2 as the chromogen. The following primary antibodies were used: Ki-67 (mouse monoclonal antibody, 1:50; Dako, Glostrup, Denmark), $p21^{Cip1}$ (mouse monoclonal antibody, 1:100; Abcam, Cambridge, UK), Cdc2 (mouse monoclonal antibody, 1:100; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), Aurora B (rabbit polyclonal antibody, 1:200; Abcam), p-Histone H3 (Ser 10 phosphorylated; rabbit polyclonal antibody, 1:50; Santa Cruz Biotechnology, Inc.), and HP1α (rabbit polyclonal antibody, 1:200; Cell Signaling Technology, Inc., Danvers, MA, USA). These antigens were selected based on our previous results (Yafune et al., 2013). Antigen retrieval was performed in an autoclave for 10 min at 121 °C in 10 mM citrate buffer (pH 6.0) for Ki-67 and p-Histone H3 and in a microwave for 10 min at 90 °C in 10 mM citrate buffer (pH 6.0) for $p21^{Cip1}$ and HP1α. Sections were counterstained with hematoxylin for microscopic examination.

2.4. Analysis of immunoreactivity

In the thyroid and urinary bladder, immunostained cells in the follicular area (thyroid) and mucosal area (urinary bladder) were counted in eight randomly selected areas per animal (four areas per tissue section) at magnifications of 400× in the former and 200× in the latter. In the forestomach, because cells immunoreactive for Ki-67, Aurora B, or HP1α were diffusely distributed in the basal cell layer, vertical length of positive cell distribution from the basement membrane was measured in 10 randomly selected areas per animal at 200× magnification. $p21^{Cip1+}$, nuclear $Cdc2^+$ or p-Histone $H3^+$ cells in the forestomach were counted in 10 randomly selected areas in the mucosa per animal at 200× magnification. In the glandular stomach, immunoreactive cells were counted in 10 randomly selected glands per animal at 200× magnification. In the colon, immunoreactive cells were counted in 10 randomly selected crypts per animal that were located close to the lamina muscularis mucosa and demonstrated a cross sectional view at 200× magnification.

Total cells were measured by counting all nuclei in each selected field in the thyroid and urinary bladder mucosa using WinROOF image analysis and measurement software (version 6.4.2., Mitani Corporation, Fukui, Japan). The percentage of immunoreactive cells was determined in each field. In the forestomach, mean vertical length of the distribution of $Ki\text{-}67^+$, Aurora B^+ or $HP1\alpha^+$ cells within the mucosa was estimated from 10 fields and expressed as vertical length (μm). $p21^{Cip1+}$, nuclear $Cdc2^+$ or p-Histone $H3^+$ cell counts were expressed as numbers per 1000 μm of epithelial layer length in each field. In the glandular stomach, mean number of immunoreactive cells of 10 glands/animal was estimated and expressed as cells per gland. In the colon, percentage of immunoreactive cells was determined in each crypt by selecting 10 glands using WinROOF image analysis and measurement software.

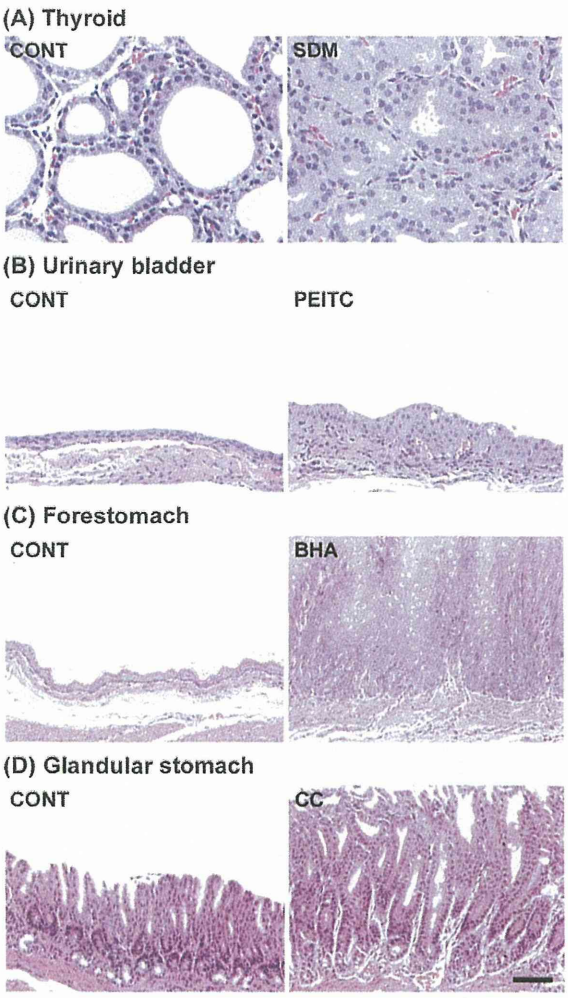

Fig. 1. Histopathological changes of the thyroid, urinary bladder, forestomach and glandular stomach in animals treated with SDM, PEITC, BHA and CC, respectively. (A) Thyroid, (B) urinary bladder, (C) forestomach and (D) glandular stomach. Bar = 50 μm (A). Bar = 100 μm (B–D).

2.5. Statistical analysis

Values for immunohistochemical cellular distribution in the thyroid, urinary bladder, forestomach, glandular stomach and colon were analyzed by Student's *t*-test with Bonferroni correction. A p values of less than 0.0056 (thyroid and urinary bladder), 0.0071 (forestomach and glandular stomach) and 0.0045 (colon) were regarded as statistically significant. Results were compared among all treatment groups and the untreated controls. Comparison between each carcinogen group in specific target organ and non-carcinogenic CL or other carcinogen groups targeting other organs was similarly performed, excluding the untreated control group from comparison. In the colon, comparison between carcinogen group targeting organ (PhIP or CDCA) and other treatment groups was similarly performed, excluding the untreated control group and PhIP or CDCA from comparison.

3. Results

3.1. Histopathological changes

Treatment with SDM resulted in scant colloidal fluid and follicular epithelial cell proliferation in the thyroid (Fig. 1A), leading to the formation of small follicles consisting of large cuboidal cells, similar to previous findings (Imai et al., 2004). Treatment with PEITC resulted in scattered foci of simple hyperplasia or papillary and nodular hyperplasia of the epithelia in the urinary bladder (Fig. 1B), similar to previous findings (Akagi et al., 2003). Treatment with BHA resulted in hyperkeratosis/parakeratosis and hyperplasia of stratified epithelia in the forestomach (Fig. 1C), similar to previous findings (Hirose et al., 1987). Treatment with CC resulted in pyloric gland hyperplasia of the glandular stomach (Fig. 1D), similar to previous findings (Hirose et al., 1999). Treatment with PhIP or CDCA did not induce any specific changes in the colon. CL as a non-carcinogenic control did not induce any changes in the epithelia of thyroid gland follicles, urinary bladder, forestomach, glandular stomach or colon. Untreated controls and carcinogens targeting other organs did not show histopathological alterations in each organ.

3.2. Immunohistochemical cellular distribution in the thyroid

In the thyroid, SDM induced a significant increase in Ki-67$^+$, p21^{Cip1+}, nuclear Cdc2$^+$ or p-Histone H3$^+$ follicular cells compared with the untreated controls, CL, BHA, CC or PhIP group (Fig. 2A–D). In contrast, BHA, CC and PhIP induced a significant decrease in Ki-67$^+$ cells compared with the untreated controls. On the other hand, CC induced a significant decrease in nuclear Cdc2$^+$ cells compared with the untreated controls. BHA induced a significant decrease in p-Histone H3$^+$ cells compared with the untreated controls. With regard to Aurora B, while SDM induced a tendency to increase in positive cells compared with the untreated controls, SDM induced a significant increase compared with CL, BHA, CC or PhIP group (Fig. 2E). With regard to HP1α, SDM did not induce a significant increase in positive cells (Fig. 2F). In comparison with the SDM group, BHA induced a significant decrease in HP1α$^+$ cells.

3.3. Immunohistochemical cellular distribution in the urinary bladder

In the urinary bladder, PEITC induced a significant increase in Ki-67$^+$, nuclear Cdc2$^+$ and p-Histone H3$^+$ cells compared with the untreated controls, CL, BHA, CC or PhIP group (Fig. 3A, C and D). With regard to p21^{Cip1}, BHA induced a significant increase in positive cells compared with the PEITC group (Fig. 3B). With regard to Aurora B, PEITC induced a significant increase in positive cells compared with the untreated controls, CL, CC or PhIP group (Fig. 3E). PEITC did not induce a significant increase in HP1α$^+$ cells (Fig. 3F).

3.4. Immunohistochemical cellular distribution in the forestomach

In the forestomach, BHA induced a significant increase in the vertical length of immunoreactive cellular distribution of Ki-67, Aurora B and HP1α from the basement membrane compared with the untreated controls, CL, CC or PhIP group (Fig. 4A, E and F). BHA also induced a significant increase in the number of nuclear Cdc2$^+$ and p-Histone H3$^+$ cells compared with the untreated controls, CL, CC or PhIP group (Fig. 4C and D). BHA did not induce a significant increase in the number of p21^{Cip1+} cells (Fig. 4B).

3.5. Immunohistochemical cellular distribution in the glandular stomach

In the glandular stomach, CC induced a significant increase in Ki-67$^+$, p21^{Cip1+}, nuclear Cdc2$^+$, *p-Histone H3$^+$ and* Aurora B$^+$ cells compared with the untreated controls, CL, BHA or PhIP group (Fig. 5A–E). However, PhIP induced a significant increase in p21^{Cip1+} cells compared with the untreated controls. CL also induced a significant increase in Aurora B$^+$ cells compared with the untreated controls. With regard to HP1α, CC did not induce a significant increase in positive cells compared with the untreated controls, whereas BHA induced a significant increase (Fig. 5F).

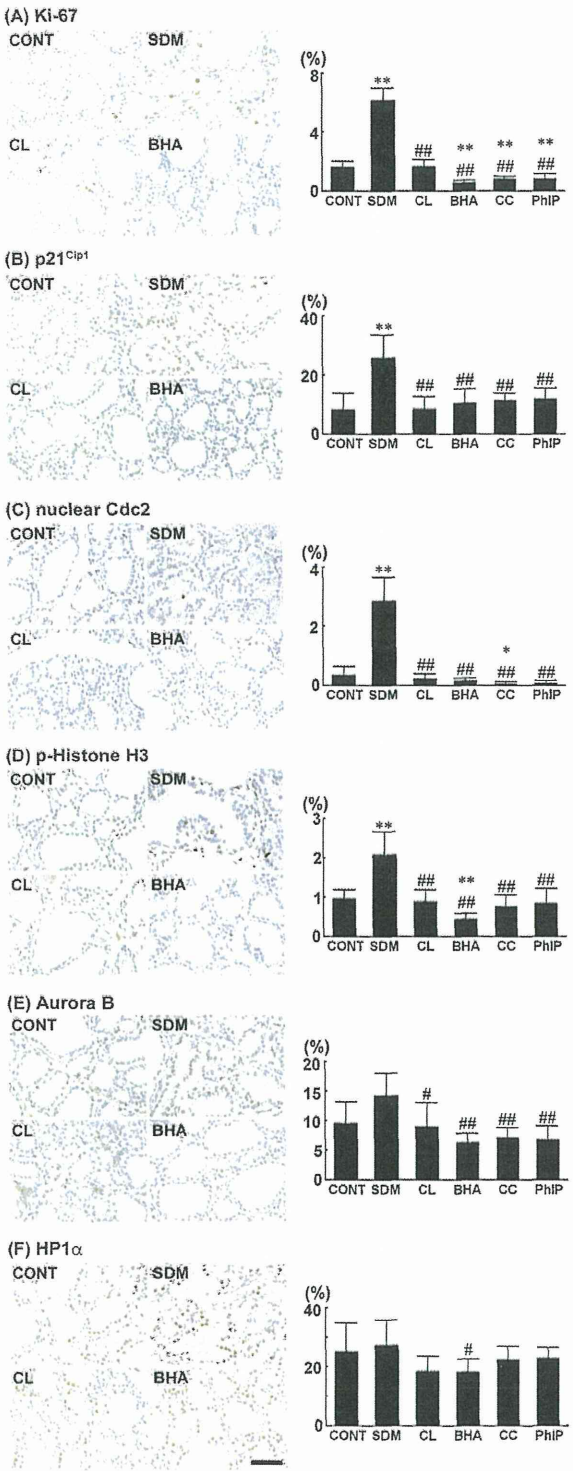

Fig. 2. Distribution of Ki-67$^+$, p21^{Cip1+}, nuclear Cdc2$^+$, p-Histone H3$^+$, Aurora B$^+$ and HP1α$^+$ cells in the thyroid. Photomicrographs show Ki-67$^+$, p21^{Cip1+}, nuclear Cdc2$^+$, p-Histone H3$^+$, Aurora B$^+$ and HP1α$^+$ cells in untreated controls and animals treated with SDM, CL or BHA. The graphs show positive cell ratios (%) of epithelial cells per total cells counted in each target organ using 10 animals per group. Values represent mean + SD. (A) Ki-67, (B) p21^{Cip1}, (C) nuclear Cdc2, (D) p-Histone H3, (E) Aurora B and (F) HP1α. Bar = 50 μm. $^{*,**}P < 0.0056$, 0.0011 vs. untreated controls (Student's *t*-test with Bonferroni correction). $^{\#,\#\#}P < 0.0056$, 0.0011 vs. SDM (Student's *t*-test with Bonferroni correction).

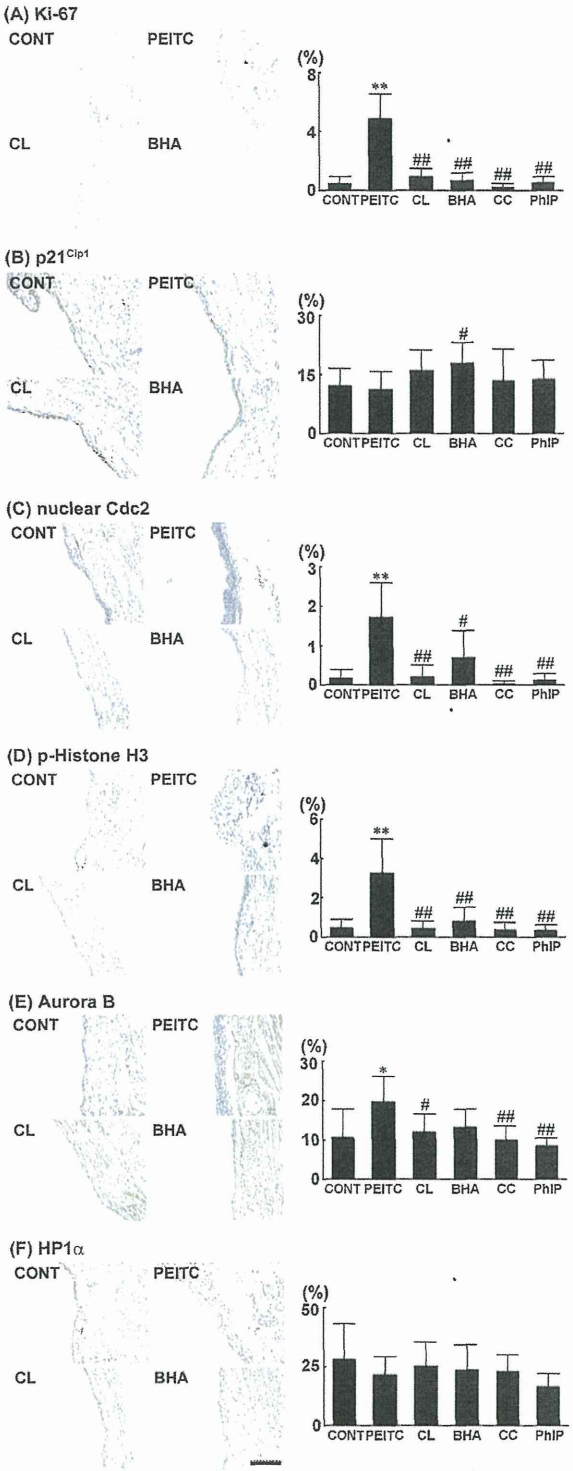

Fig. 3. Distribution of Ki-67$^+$, p21^{Cip1+}, nuclear Cdc2$^+$, p-Histone H3$^+$, Aurora B$^+$ and HP1α$^+$ cells in the urinary bladder. Photomicrographs show Ki-67$^+$, p21^{Cip1+}, nuclear Cdc2$^+$, p-Histone H3$^+$, Aurora B$^+$ and HP1α$^+$ cells in untreated controls and animals treated with PEITC, CL or BHA. The graphs show positive cell ratios (%) of epithelial cells per total cells counted in each target organ using 10 animals per group. Values represent mean + SD. (A) Ki-67, (B) p21^{Cip1}, (C) nuclear Cdc2, (D) p-Histone H3, (E) Aurora B and (F) HP1α. Bar = 100 μm. $^{*,**}P < 0.0056$, 0.0011 vs. untreated controls (Student's *t*-test with Bonferroni correction). $^{\#,\#\#}P < 0.0056$, 0.0011 vs. PEITC (Student's *t*-test with Bonferroni correction).

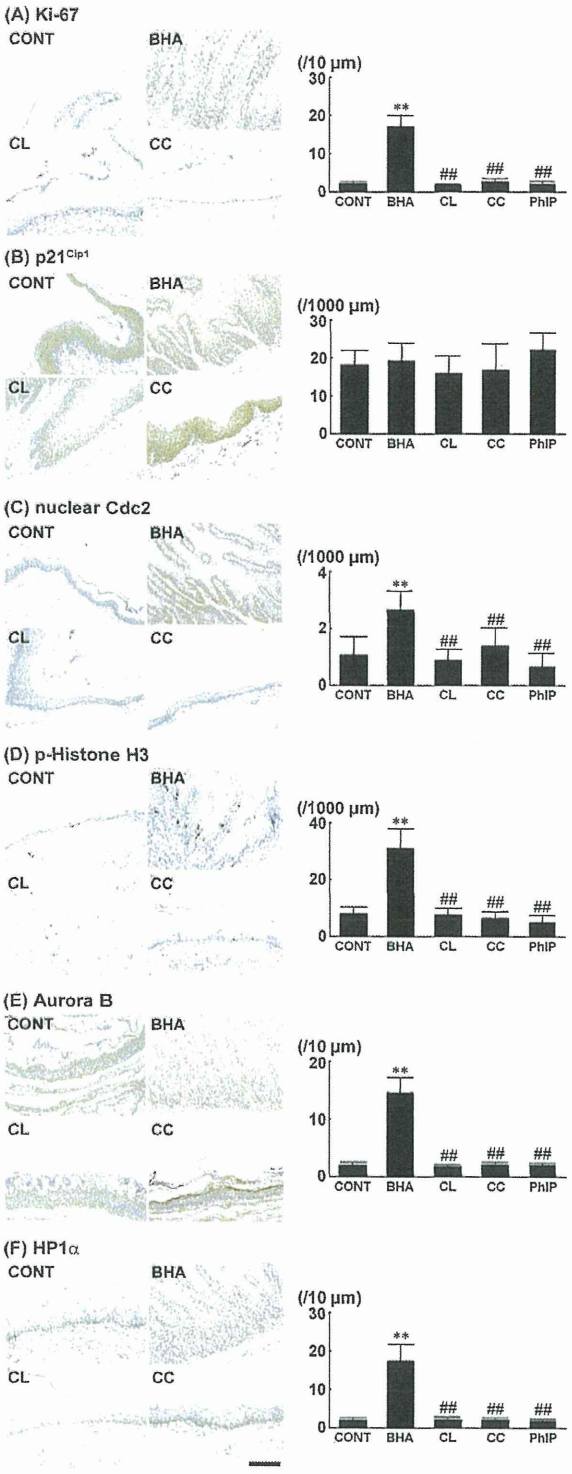

Fig. 4. Distribution of Ki-67^{+}, p21^{Cip1+}, nuclear Cdc2^{+}, p-Histone H3^{+}, Aurora B^{+} and HP1α^{+} cells in the forestomach. Photomicrographs show Ki-67^{+}, p21^{Cip1+}, nuclear Cdc2^{+}, p-Histone H3^{+}, Aurora B^{+} and HP1α^{+} cells in untreated controls and animals treated with BHA, CL or CC. (A), (E) and (F) show vertical length of positive cell distribution from the basement membrane per unit area using 10 animals per group. (B), (C) and (D) show mean number of positive cells per unit horizontal length (1000 μm). Values represent mean + SD. (A) Ki-67, (B) p21^{Cip1}, (C) nuclear Cdc2, (D) p-Histone H3, (E) Aurora B and (F) HP1α. Bar = 100 μm. $^{**}P < 0.0014$ vs. untreated controls (Student's *t*-test with Bonferroni correction). $^{\#\#}P < 0.0014$ vs. BHA (Student's *t*-test with Bonferroni correction).

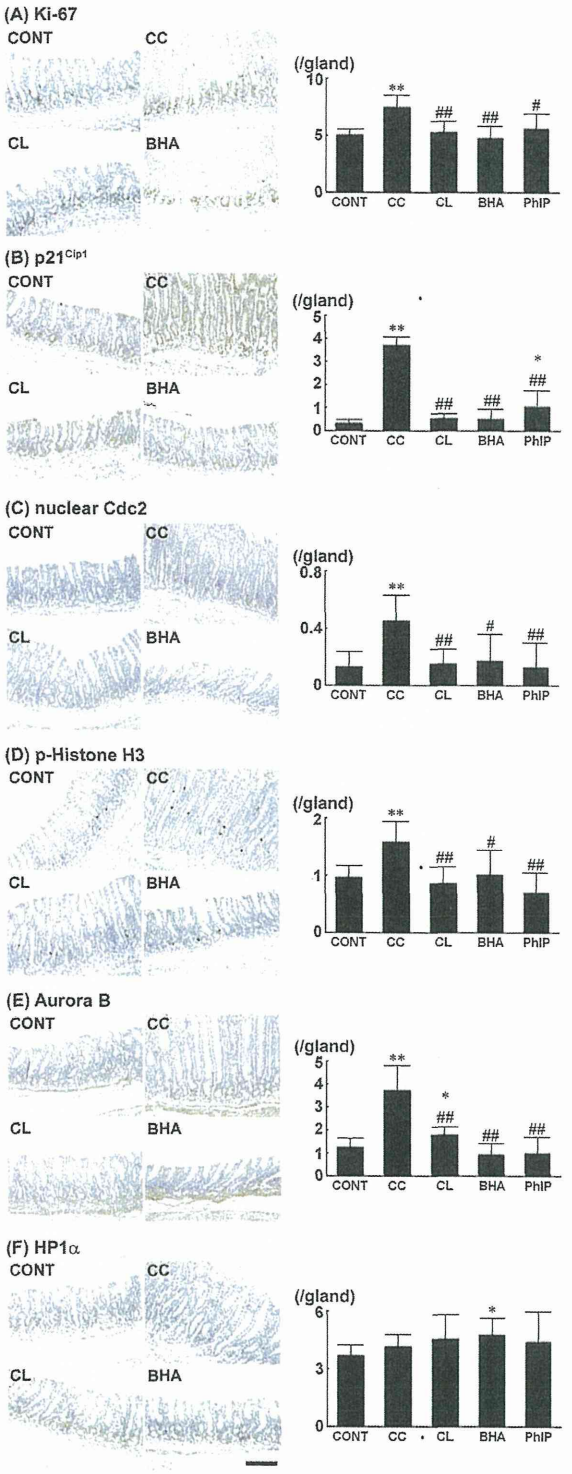

Fig. 5. Distribution of Ki-67^{+}, p21^{Cip1+}, nuclear Cdc2^{+}, p-Histone H3^{+}, Aurora B^{+} and HP1α^{+} cells in the glandular stomach. Photomicrographs show Ki-67^{+}, p21^{Cip1+}, nuclear Cdc2^{+}, p-Histone H3^{+}, Aurora B^{+} and HP1α^{+} cells in untreated controls and animals treated with CC, CL or BHA. The graphs show mean number of positive cells per gland using 10 animals per group. Values represent mean + SD. (A) Ki-67, (B) p21^{Cip1}, (C) nuclear Cdc2, (D) p-Histone H3, (E) Aurora B and (F) HP1α. Bar = 100 μm. $^{*,**}P < 0.0071, 0.0014$ vs. untreated controls (Student's *t*-test with Bonferroni correction). $^{\#,\#\#}P < 0.0071$, 0.0014 vs. CC (Student's *t*-test with Bonferroni correction).

3.6. Immunohistochemical cellular distribution in the colon

In the colon, PhIP and CDCA did not induce a significant increase in any marker in the colon compared with the untreated controls (Fig. 6A–F). In comparison with the PhIP or CDCA group, no marker induced a significant change.

4. Discussion

In the present study, we found carcinogens that specifically targeted the thyroid, urinary bladder, forestomach and glandular stomach induced increases in Ki-67$^+$, nuclear Cdc2$^+$, p-Histone H3$^+$ and Aurora B$^+$ cells in their respective target organs. However, some carcinogens lacking induction of cell proliferation as estimated by the number of Ki-67$^+$ cells in the target organ, CDCA and PhIP in the colon did not increase all tested markers. We also previously found that carcinogens that evoked cell proliferation after 28 days of treatment increased apoptotic cells and cells expressing ubiquitin D (Ubd) at the G_2 phase, irrespective of target organs examined (Taniai et al., 2012). These results may suggest a sequential change of cell cycle aberration at the initial step of carcinogenesis facilitating target cell proliferation.

Experimentally, short-term treatment with carcinogens often induces proliferation of target cells (Akagi et al., 2003; Mally and Chipman, 2002). In the present study, SDM, PEITC, BHA and CC increased cell proliferation activity after 28 days of treatment. The hypothyroidism-related mechanism of SDM involves a decrease in the serum levels of T_3 and T_4, causing suppression of a negative feedback through the pituitary and an increase in the levels of serum thyroid-stimulating hormone (TSH) in rats (Imai et al., 2004). TSH then stimulates thyroid functions, including growth and proliferation of follicular cells as a link to carcinogenesis (Hard, 1998). PEITC induces cell proliferation of urinary epithelial cells after short-term administration because of cytotoxic oxidative DNA damage (Akagi et al., 2003). BHA induces cell proliferation of forestomach epithelium (Cantoreggi et al., 1993). CC induces cell proliferation in response to gastric epithelial erosion/ulceration (Hirose et al., 1999). In contrast, none of the carcinogens induced cell proliferation activity in non-target organs. These results suggest that there is a group of carcinogens, irrespective of target organs, that have the potential to induce proliferation of target cells in a 28-day treatment scheme. On the other hand, it has been reported that BHA enhanced cell proliferation in the esophagus, glandular stomach, small intestine and large intestine, in addition to the forestomach after oral treatment for 14 days in rats (Verhagen et al., 1990). However, BHA did not increase Ki-67$^+$ proliferating cells in the glandular stomach and colon in the present study. It has been previously reported that carcinogen-induced cell proliferation activity changes during the administration period (Akagi et al., 2003; Hirose et al., 1999). Therefore, it is possible that BHA no longer induces cell proliferation in the glandular stomach and colon after 28 days of treatment.

Cdc2 and cyclin B form the cyclin B–Cdc2 complex, which initiates the G_2/M transition, and nuclear localization of Cdc2 represents the active isoform entering at the M phase (Chan et al., 1999; Kawamoto et al., 1997). p-Histone H3 is crucial for chromosome condensation and segregation during mitosis (Nowak and Corces, 2004). Aurora B, a chromosomal passenger protein, plays a role in spindle assembly and chromosome segregation in mitosis (Meraldi et al., 2004). In the present study, SDM, PEITC, BHA and CC increased immunoreactive cells for nuclear Cdc2, p-Histone H3 and Aurora B, compared with the untreated controls, CL-treated controls and carcinogens targeting other organs. It has been demonstrated that prometaphase-arrested cells overexpress Cdc2 (Choi et al., 2011).

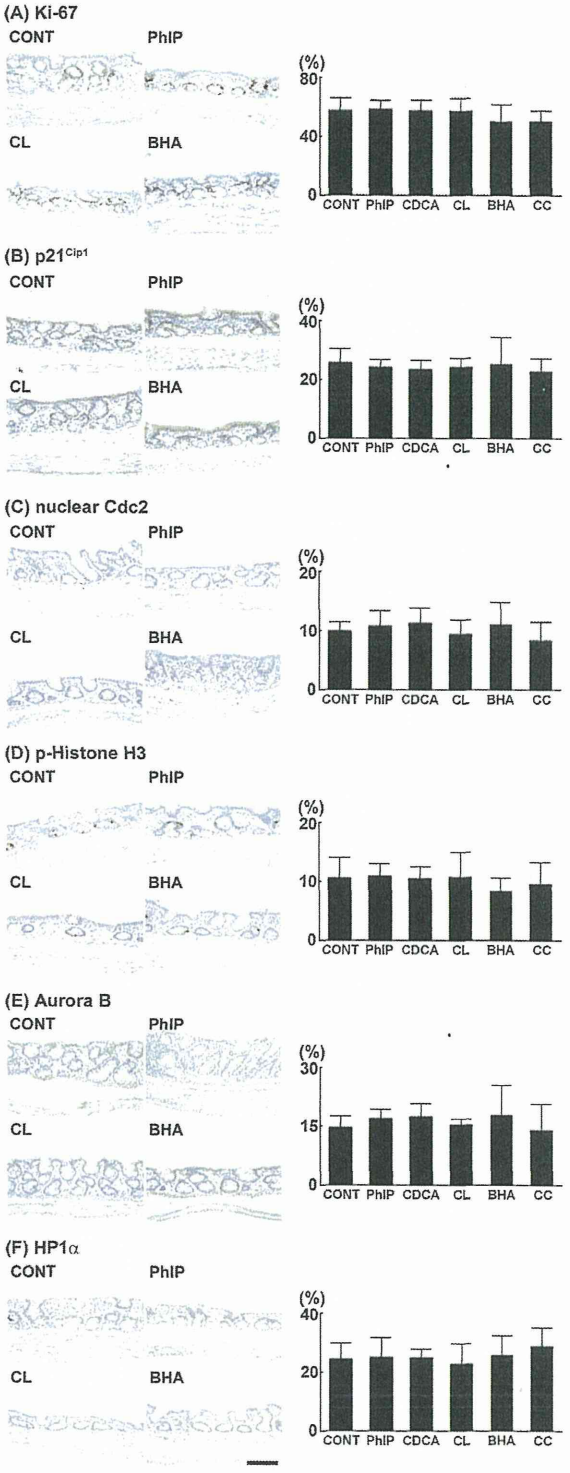

Fig. 6. Distribution of Ki-67$^+$, p21^{Cip1+}, nuclear Cdc2$^+$, p-Histone H3$^+$, Aurora B$^+$ and HP1α$^+$ cells in the colon. Photomicrographs show Ki-67$^+$, p21^{Cip1+}, nuclear Cdc2$^+$, p-Histone H3$^+$, Aurora B$^+$ and HP1α$^+$ cells in the colon in untreated controls and animals treated with PhIP, CL or BHA. The graphs show positive cell ratios (%) of epithelial cells per total cells counted in crypts using 10 animals per group. Values represent mean + SD. (A) Ki-67, (B) p21^{Cip1}, (C) nuclear Cdc2, (D) p-Histone H3, (E) Aurora B and (F) HP1α. Bar = 100 μm.

Overexpression of Aurora B causes chromosomal instability in various cancer cells (Qi et al., 2007) and increases in p-Histone H3 as a result of overexpression of Aurora B that contributes to chromosome number instability (Ota et al., 2002). These observations

suggest carcinogens that evoke cell proliferation increased cellular populations arrested at the M phase or showing chromosomal instability, irrespective of target organs. As we discussed previously, an aberrant increase of Ubd^+ cells at G_2 phase by carcinogens evoking cell proliferation in the target organ after a 28-day treatment may suggest an increase of the cellular population staying at the M phase (Taniai et al., 2012), because Ubd functions on the disruption of the spindle checkpoint at the M phase (Lim et al., 2006).

$p21^{Cip1}$ is one of the cyclin-dependent kinase (CDK) inhibitors that plays a role in G_1 checkpoint (Sherr and Roberts, 1995). Increased expression of this protein may link to G_1/S cell cycle arrest and apoptosis dependently with p53 (Liu et al., 2011). We previously found hepatocarcinogens that evoke liver cell proliferation evoked concomitant liver cell apoptosis that may link to p53 activation after 28-day treatment in rats (Yafune et al., 2013). In that study, we also found that all hepatocarcinogens tested increased $p21^{Cip1+}$ liver cells independently with cell proliferation activity (Yafune et al., 2013). Therefore, $p21^{Cip1}$ was expected to be an early prediction marker of carcinogens irrespective of the induction potential of target cell proliferation. However, in the present study, PEITC and BHA that evoked cell proliferation did not increase $p21^{Cip1+}$ cells in each target organ. It was previously reported that there was no obvious relationship between the expression of $p21^{Cip1}$ and cell proliferation activity in the development of proliferative lesions during carcinogenesis (Lu et al., 1999).

With regard to HP1α, only BHA induced an increase in immunoreactive cells in the forestomach epithelia, whereas SDM, PEITC and CC that induced cell proliferation did not increase $HP1\alpha^+$ cells in their target organ. It has been reported that HP1α plays a major role in chromosomal segregation during mitosis (Obuse et al., 2004), and phosphorylation of HP1α is necessary for genome stability (Hiragami-Hamada et al., 2011). Therefore, a phosphorylated cell population of HP1α rather than total cell population expressing HP1α may reflect a functional relationship of this protein for chromosomal maintenance.

We previously demonstrated that colon carcinogens (PhIP and CDCA) did not alter cellular distribution of proliferation markers, apoptosis index and G_2/M phase proteins (Taniai et al., 2012). In the present study, PhIP and CDCA also did not alter cellular distribution of the selected markers in colonic epithelia in accordance with the previous study. PhIP at 400 ppm in diet slightly induced colonic mucosal cell proliferation after 8-week administration, but the proliferation index was not increased after 4-week administration (Ochiai et al., 1996). It has been previously reported that CDCA administration for 2 weeks could induce cell proliferation of colonic epithelial cells after initiation with azoxymethane (Sutherland and Bird, 1994). However, CDCA without the initiation treatment only increased cell proliferation at low doses. CDCA at 1000 ppm in diet did not increase colonic mucosal cell proliferation in the present study. Therefore, it is possible that these colon carcinogens would not induce aberrant expression of M phase proteins in conjunction with activation of cell proliferation in a 28-day treatment scheme.

In conclusion, cell proliferation-evoking carcinogens in a 28-day treatment scheme can induce aberrant activation of M phase proteins reflecting cell cycle disruption, irrespective of target organs, which suggests an outcome of an increase in cell populations arrested at M phase or showing chromosomal instability. Carcinogens lacking proliferative activity did not have these effects. These results suggest that Ki-67 as a cell proliferation marker and nuclear Cdc2, p-Histone H3 and Aurora B as M phase proteins might functions as a rapid screening battery of biomarkers for carcinogens that exhibit high proliferative activity after a 28-day treatment in many target organs.

Conflict of interest statement

The authors disclose that there are no competing financial interests that could inappropriately influence the outcome of this study.

Acknowledgements

The authors thank Mrs. Shigeko Suzuki for her technical assistance in preparing the histological specimens. This work was supported by Health and Labor Sciences Research Grants (Research on Food Safety) from the Ministry of Health, Labor and Welfare of Japan.

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