Table 3 Final body and liver weights of rats after 28-day treatment with hepatocarcinogens or hepatotoxicants

Group	No. of animals examined	Final body weight (g)	Liver weight	
			Absolute (g)	Relative (g/100 g BW)
Untreated controls	10	210±16[a]	7.93±0.87	3.68±0.17
TAA	10	141±13**	7.08±0.87*	4.94±0.24**
FB	10	208±14	8.62±0.68	4.04±0.14**
PBO	10	164±10**	11.89±0.77**	7.16±0.18**
MEG	10	175±13**	10.09±0.89**	5.68±0.31**
APAP	10	173±9**	7.17±0.55*	4.07±0.15**
ANIT	10	111±5**	6.43±0.44**	5.70±0.21**

* $P < 0.05$ versus untreated controls

** $P < 0.01$ versus untreated controls

[a] Mean ± SD

liver weights and real-time RT-PCR analyses, numerical data of the treatment groups were compared with those of the untreated controls. In case of the immunohistochemical analyses, numerical data were compared among all treatment groups and the untreated controls. Comparison between the APAP or ANIT and other treatment groups was similarly performed, excluding the untreated controls from comparison.

Results

Final body and liver weights

The final body and liver weights are shown in Table 3. As compared with the untreated controls, final body weights were significantly decreased in the TAA, PBO, MEG, APAP, and ANIT groups. The absolute liver weights of rats in the TAA, APAP, and ANIT groups were significantly lower than those of the untreated controls. The absolute liver weights of rats in the PBO and MEG groups were significantly higher than those of the untreated controls, but those of the FB group were not significantly changed. The relative liver weights of all treatment groups were significantly higher than those of the untreated controls.

Histopathological changes

Treatment with TAA induced diffuse liver cell cytomegaly often associated with anisokaryosis, aberrant mitosis, and apoptosis, as previously reported (Clawson et al. 1992). Bile duct proliferation and oval cell proliferation accompanied with mild interstitial fibrosis were evident in the periportal area. FB treatment induced centrilobular liver cell hypertrophy characterized by a marked increase in smooth endoplasmic reticulum, as previously reported (Shoda et al. 1999). Periportal liver cells showed cytomegaly associated with anisokaryosis and scattered mitoses. PBO treatment induced diffuse liver cell hypertrophy associated with cytoplasmic ground appearance and moderate nuclear enlargement, as previously reported (Muguruma et al. 2007). MEG treatment resulted in diffuse distribution of cytomegalic liver cells associated with anisokaryosis, as previously reported (NTP 2000). Centrilobular liver cell necrosis was also scattered. APAP treatment resulted in cytoplasmic eosinophilia and ground glass appearance of liver cells and ANIT treatment resulted in periportal bile duct proliferation, and scattered focal liver cell necrosis and microgranulomas, as previously reported (NTP 1993; Rees et al. 1962). However, neither APAP nor ANIT induced karyomegaly or cytomegaly in the liver cells.

Global gene expression changes after TAA treatment

Among the 26,208 gene targets identified in the microarray analysis, 2,888 genes showed altered expression in liver tissue after 28 days of TAA treatment compared to the untreated controls (1,681 genes were upregulated and 1,207 genes were downregulated, Online Resource 1). Most of the identified upregulated genes were related to cell proliferation, apoptosis, cell cycle, or intracellular signaling systems. Upregulated genes with apparent cell cycle function are listed in Table 4. The data discussed in this study have been deposited in NCBIs Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo/) and are accessible through GEO Series accession number GSE43066.

Real-time RT-PCR analysis

Expression levels of representative cell cycle-related genes selected from those listed in Table 4 were determined by real-time RT-PCR in the TAA and FB groups and compared with the untreated controls (Table 5). mRNA levels of all examined genes (*Cdkn2b, Cdkn1a, Ccnd1, Ccna2, Ccne2, Cdr2, Wee1, Tpx2, Gadd45a, Cdk1, Ccnb1, Aurkb,* and *Aurka*) were significantly increased in the TAA group as compared with the untreated controls. In addition, mRNA levels of *Cdkn2b, Cdkn1a, Ccnd1, Ccne2, Cdr2,* and *Cdk1* were also significantly increased in the FB group as compared with the untreated controls.

Cell proliferative activity and apoptosis

The nuclear antigen Ki-67 is a cell proliferation marker that is expressed in cells during the G_1 to M phase of the cell cycle (Scholzen and Gerdes 2000). The number of Ki-67$^+$ cells was significantly increased in the TAA, FB, and MEG groups as compared with the untreated controls, as previously reported (Taniai et al. 2012; Online Resource 2, Fig. s1A). A significant increase in Ki-67$^+$ cells was observed in the TAA and MEG groups as compared with the non-carcinogen groups (APAP and ANIT), while there was no difference in the number of Ki-67$^+$ cells in FB group compared with both non-carcinogen groups. PBO did not increase the number of Ki-67$^+$ proliferating cells.

TUNEL$^+$ cells were significantly increased in the TAA and MEG groups as compared with the untreated controls and non-carcinogen groups, as previously reported (Taniai et al. 2012; Online Resource 2, Fig. s1B). FB induced a significant increase in TUNEL$^+$ cells as compared with the APAP group. PBO did not increase the number of TUNEL$^+$ cells.

Immunoreactivity of G_1/S phase transition proteins

$p21^{Cip1}$ is a one of the Cyclin-dependent kinase (CDK) inhibitors that play a role in the G_1 checkpoint (Sherr and Roberts 1995). There was nuclear immunoreactivity for $p21^{Cip1}$ in the liver cells of untreated controls (Fig. 1a). The number of cells that was immunoreactive for $p21^{Cip1}$ was significantly increased in all carcinogen-treated groups as compared with the untreated controls and both non-carcinogen groups (Fig. 1a).

$p16^{Ink4a}$ and $p27^{Kip1}$, CDK inhibitors playing a role in the facilitation of the G_1 cell cycle arrest by binding to CDKs (Sherr and Roberts 1995), showed weak to moderate nuclear immunoreactivity in the liver cells of untreated controls. Strong nuclear and cytoplasmic immunoreactivity for $p16^{Ink4a}$ were found in the TAA group. Conversely, animals treated with other carcinogens or non-carcinogens only showed nuclear immunoreactivity. However, the number of immunoreactive cells was mostly unchanged by these treatments (Online Resource 2, Fig. s2A). The number of $p27^{Kip1+}$ cells did not statistically change in any of the carcinogen groups as compared with the untreated controls and non-carcinogen groups (Online Resource 2, Fig. s2B).

Immunoreactivity of G_2/M transition proteins

Cdc2, a molecule that drives the G_2/M transition in coordination with cyclin B1 (Chan et al. 1999), showed nuclear and/or cytoplasmic immunoreactivity in the liver cells of the untreated controls with the antibody recognizing both the phosphorylated and non-phosphorylated isoforms used here. Because Cdc2 is transported into the nucleus together with cyclin B1 upon activation (Chan et al. 1999), we counted cells showing nuclear immunoreactivity. Expression of nuclear Cdc2$^+$ cells significantly increased in the TAA, FB, and MEG groups as compared with the untreated controls and both non-carcinogen groups. However, PBO did not increase the number of nuclear Cdc2$^+$ cells (Fig. 1b).

Phospho-Wee1, acting at the G_2/M transition (Hashimoto et al. 2006), showed weak-to-moderate nuclear immunoreactivity in the liver cells of untreated controls. However, there were no specific expression changes in response to carcinogen treatment as compared with untreated controls or non-carcinogen groups (Online Resource 2, Fig. s2C).

Immunoreactivity of M phase proteins

Aurora B and Incenp, an interaction partner during mitosis (Ruchaud et al. 2007), were immunolocalized in the nuclei of the liver cells of untreated controls. Aurora B-immunoreactive cells were significantly increased in all carcinogen groups as compared with the untreated controls and the APAP group. In comparison with the ANIT group, Aurora B-immunoreactive cells significantly increased after treatment with FB, PBO, and MEG (Fig. 1c). Incenp-immunoreactive cells were significantly increased in all carcinogen groups as compared with the untreated controls and the ANIT group. However, APAP significantly increased Incenp$^+$ cells similar to the carcinogen groups (Fig. 1d). Phosphorylated-Histone H3 (p-Histone H3), the phosphorylated active isoform by Aurora B-kinase activity, causes heterochromatin protein 1α (HP1α) dissociation from heterochromatin, both acting at the early M phase (Hirota et al. 2005). Both p-Histone H3 and HP1α showed nuclear immunolocalization in the untreated controls. p-Histone H3$^+$ cells were significantly increased in the carcinogen groups as compared with the untreated controls and the APAP group. In comparison with the ANIT group, p-Histone H3 immunoreactive cells were significantly increased in the TAA, FB, and MEG groups (Fig. 1e). The number of HP1α^+ cells was significantly increased in the TAA, FB, and MEG groups as compared with the untreated controls. A significant increase in HP1α^+ cells was observed in the TAA and MEG groups as compared with the non-carcinogen groups, while the number of HP1α^+ cells in the FB group was statistically not different from those of the non-carcinogen groups. PBO did not increase HP1α^+ cells (Fig. 1f).

Expression of p53 and downstream Mdm2

Tp53 mRNA levels were significantly increased in the TAA group as compared with the untreated controls.

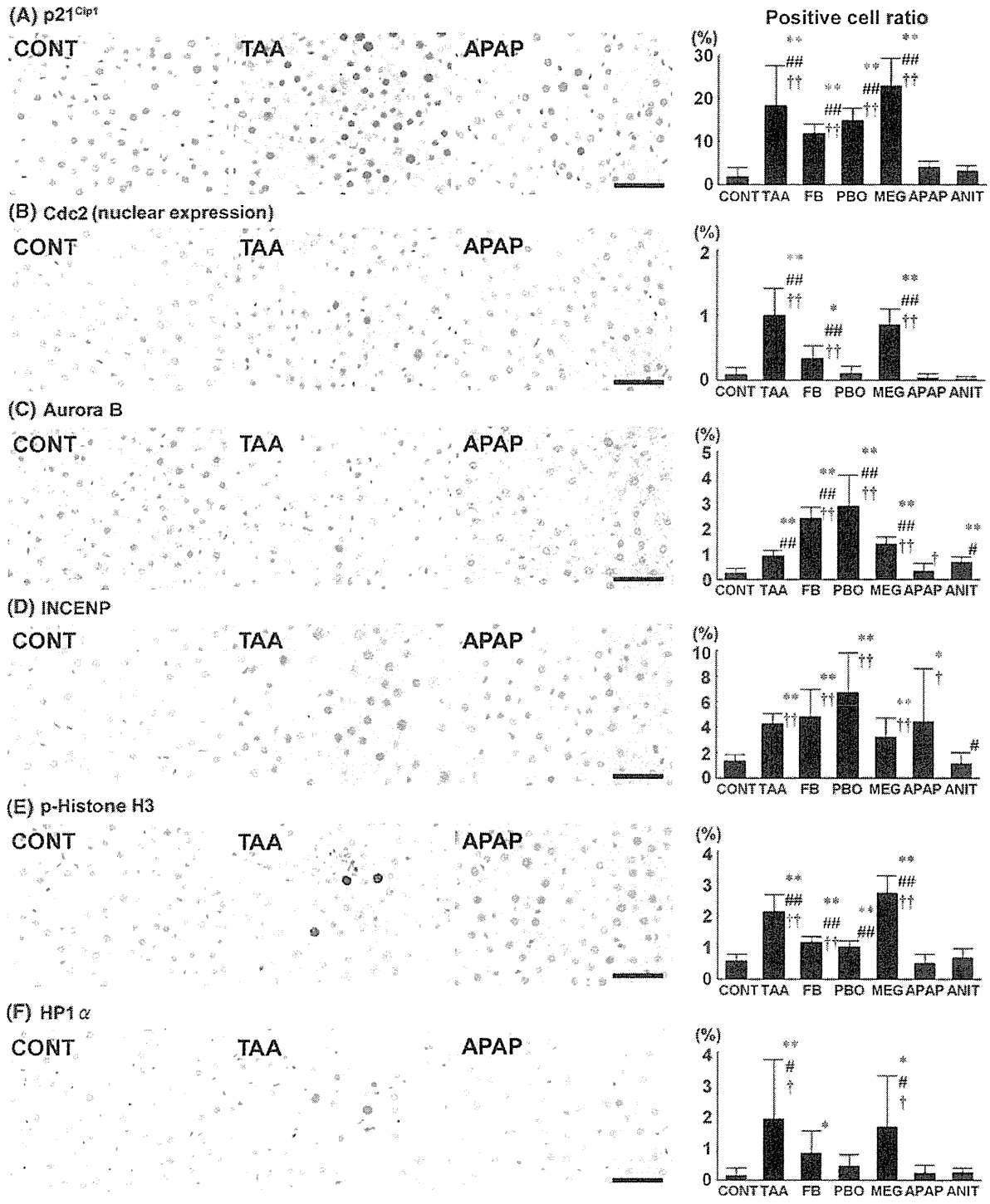

Fig. 1 Immunohistochemical cellular distribution of cell cycle proteins in liver cells after 28-day treatment with hepatocarcinogens or non-carcinogens in rats. Photomicrographs show distributions of $p21^{Cip1}$, nuclear Cdc2, Aurora B, Incenp, p-Histone H3, and HP1α immunoreactive cells in the livers of representative cases of an untreated control and animals treated with TAA or APAP. The graphs show positive cell ratios (%) of liver cells per total cells counted using 10 animals in each group. Values are presented as mean + SD (**a**) $p21^{Cip1}$, (**b**) Cdc2, (**c**) Aurora B, (**d**) Incenp, (**e**) p-Histone H3, and (**f**) HP1α. Magnification:×400 (*Bar* = 50 μm). *, ** $P < 0.05$, 0.01 versus untreated controls (Dunnett's or Steel's test). #, ## $P < 0.05$, 0.01 versus APAP (Dunnett's or Steel's test). †, †† $P < 0.05$, 0.01 versus ANIT (Dunnett's or Steel's test)

Table 4 Representative cell cycle-related genes with known functional annotations that were upregulated in the livers of rats treated with TAA (≥twofold)

Accession No.	Gene title	Symbol	TAA
XM_001054052	Anaphase promoting complex subunit 4	Anapc4	2.30
NM_153296	Aurora kinase A	Aurka	2.22
NM_053749	Aurora kinase B	Aurkb	4.03
XM_001080790	Cancer susceptibility candidate 5	Casc5	2.71
NM_053702	Cyclin A2	Ccna2	2.26
NM_171991	Cyclin B1	Ccnb1	4.16
NM_001009470	Cyclin B2	Ccnb2	2.45
NM_171992	Cyclin D1	Ccnd1	2.54
XM_001077331	Cyclin E1	Ccne1	3.80
XM_001064075	Cyclin E2	Ccne2	3.94
NM_012923	Cyclin G1	Ccng1	6.18
NM_019296	Cyclin-dependent kinase 1	Cdc2	2.52
XM_001068286	Cell division cycle associated 2	Cdca2	5.10
NM_001007648	Cell division cycle associated 3	Cdca3	2.93
NM_001025693	Cell division cycle associated 7	Cdca7	7.72
NM_001025050	Cell division cycle associated 8	Cdca8	2.92
NM_001012035	Cyclin-dependent kinase-like 2 (CDC2-related kinase)	Cdkl2	2.06
NM_080782	Cyclin-dependent kinase inhibitor 1A	Cdkn1a	5.96
NM_130812	Cyclin-dependent kinase inhibitor 2B (p15, inhibits CDK4)	Cdkn2b	2.52
NM_001025682	Cerebellar degeneration-related 2	Cdr2	5.90
XM_001069485	Centromere protein A	Cenpa	2.72
XM_001077739	Centromere protein E	Cenpe	2.78
NM_001008366	Centromere protein N	Cenpn	3.60
NM_001014215	Centromere protein Q	Cenpq	2.28
NM_001025646	Centrosomal protein 55	Cep55	2.20
NM_001017470	Centrosomal protein 70	Cep70	2.88
XM_001067027	Centrosomal protein 76	Cep76	3.34
XM_001076228	Centrosomal protein 135	Cep135	2.58
NM_080400	CHK1 checkpoint homolog (S. pombe)	Chek1	2.60
XM_001058264	Claspin homolog (Xenopus laevis)	Clspn	2.29
XM_001073486	Disks, large (Drosophila) homolog-associated protein 5	Dlgap5	2.68
XM_001070442	Excision repair cross-complementing rodent repair deficiency complementation group 6-like	Ercc6 l	2.11
XM_001067790	Extra spindle poles like 1 (S. cerevisiae)	Espl1	2.30
XM_001065873	F-box protein 5	Fbxo5	2.06
XM_001075601	Fizzy/cell division cycle 20 related 1 (Drosophila)	Fzr1	2.20
NM_024127	Growth arrest and DNA-damage-inducible, alpha	Gadd45a	2.54
NM_001008321	Growth arrest and DNA-damage-inducible, beta	Gadd45b	2.73
XM_001078275	G-2 and S-phase expressed 1	Gtse1	14.53
XM_001074188	Inner centromere protein	Incenp	3.37
XM_001065116	Kinesin family member 2A	Kif2a	4.79
NM_001085369	Kinesin family member 2C	Kif2c	2.10
XM_001061764	Kinesin family member 20A	Kif20a	2.43
XM_001070728	Minichromosome maintenance complex component 3	Mcm3	2.94
XM_001068436	Similar to mcdc21 protein; minichromosome maintenance complex component 4	Mcm4	2.50
NM_012603	Myelocytomatosis oncogene	Myc	3.30
XM_001055166	NIMA (never in mitosis gene a)-related expressed kinase 2	Nek2	2.07
NM_182953	NIMA (never in mitosis gene a)-related kinase 6	Nek6	2.34
NM_177931	Origin recognition complex, subunit 1-like (yeast)	Orc1 l	2.10

Table 4 continued

Accession No.	Gene title	Symbol	TAA
NM_199092	Origin recognition complex, subunit 4-like (yeast)	Orc4 l	2.53
NM_001033690	Origin recognition complex, subunit 6 like (yeast)	Orc6 l	2.19
NM 017198	p21 protein (Cdc42/Rac)-activated kinase 1	Pak1	2.90
NM_017100	Polo-like kinase 1 (Drosophila)	Plk1	3.24
NM_031821	Polo-like kinase 2 (Drosophila)	Plk2	3.41
NM_001007754	Ras association (RalGDS/AF-6) domain family member 1	Rassf1	2.43
XM_001055763	Retinoblastoma-like 1 (p107)	Rbl1	2.60
XM_001077474	SPC24, NDC80 kinetochore complex component, homolog (S. cerevisiae)	Spc24	4.70
NM_001009654	SPC25, NDC80 kinetochore complex component, homolog (S. cerevisiae)	Spc25	5.73
NM_022183	Topoisomerase (DNA) II alpha	Top2a	2.20
NM_001107790	TPX2, microtubule-associated, homolog (Xenopus laevis)	Tpx2	3.18
NM_001012742	Wee 1 homolog (S. pombe)	Wee1	6.38
NM_019376	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, gamma polypeptide	Ywhag	2.30

Values are fold change with the expression level in the untreated control group set as 1

mRNA levels of *Mdm2*, a protein regulated by *Tp53*, were significantly increased in both TAA and FB groups as compared with the untreated controls (Fig. 2a).

Immunohistochemically, p53 showed nuclear immunolocalization in the untreated controls. Immunoreactive cells for p53 were significantly increased in the TAA, FB, and MEG groups as compared with the untreated controls and the non-carcinogen groups (Fig. 2b). However, PBO did not increase the number of $p53^+$ cells.

Real-time RT-PCR analysis of Ndrg1 and Klf6

Gene expression levels of N-myc downstream regulated gene 1 (*Ndrg1*) and Kruppel-like factor 6 (*Klf6*) were investigated in all hepatocarcinogen groups and compared with those of the untreated controls (Fig. 3). Transcript levels of *Ndrg1* significantly increased in the TAA group as compared with the untreated controls. Transcript levels of *Klf6* significantly increased in the TAA, PBO, and MEG groups as compared with the untreated controls. Transcript levels of *Klf6* were also non-significantly increased in the FB group (Fig. 3).

Discussion

We used microarrays to profile gene expression in the livers of rats treated with TAA for 28 days and found fluctuations in transcript levels of cell cycle-related genes. We then investigated the immunohistochemical cellular distribution of cell cycle proteins and reviewed the results along with the cell proliferation activity and apoptotic cell distribution that we previously studied in the livers of rats treated with hepatocarcinogens or non-hepatocarcinogenic hepatotoxicants (Taniai et al. 2012). We found that all hepatocarcinogens studied, irrespective of their cytomegaly inducing potential, increased cellular distribution of the G_1/S checkpoint protein $p21^{Cip1}$ and of the M phase proteins Aurora B and Incenp. We also found that hepatocarcinogens that induced high cell proliferation activity by increasing the number of $Ki\text{-}67^+$ cells induced increases in the cellular distribution of p53 and the M phase-related proteins nuclear Cdc2, p-Histone H3, and HP1α. These results suggest that hepatocarcinogens increased cell populations in the G_1/S checkpoint or M phase, and hepatocarcinogens that induced high cell proliferation activity also increased cell populations facilitating M phase arrest.

Repeated treatment of rats with chemical carcinogens often results in target cell proliferation (Tanaka et al. 2000; Lock and Hard 2004). We recently found that carcinogens that caused high cell proliferation activity, irrespective of the target organ, increased the population of cells co-expressing Topo IIα and ubiquitin D (Ubd) (Taniai et al. 2012). Topo IIα functions at the G_2/M transition (Wang et al. 2008), and overexpression of Ubd leads to chromosomal instability through reduction of kinetochore localization of the spindle assembly checkpoint protein Mad2 (Lim et al. 2006; Herrmann et al. 2007). In the present study, we found an increase in nuclear Cdc2-expressing liver cells similar to that of $Ki\text{-}67^+$ cells after treatment with three of the four hepatocarcinogens (TAA, FB and MEG). 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 (Kawamoto et al. 1997; Chan et al. 1999). We previously found an increase in $Cdc2^+$ cells parallel to that of $Ki\text{-}67^+$ cells in the proliferative lesions in a

Table 5 Validations in transcript levels measured by real-time RT–PCR in the livers of rats treated with TAA or FB

Gene symbol	Real-time RT-PCR normalized by *Actb*	
	TAA[a]	FB[a]
Cdkn2b	4.32 ± 0.20[b]**	2.03 ± 0.57**
Cdkn1a	3.49 ± 1.02**	2.32 ± 0.69**
Ccnd1	2.73 ± 0.52**	1.79 ± 0.48*
Ccna2	3.06 ± 0.61**	1.56 ± 0.52
Ccne2	6.60 ± 2.11**	1.48 ± 0.33*
Cdr2	7.94 ± 2.01**	1.44 ± 0.25*
Wee1	4.99 ± 2.83*	1.18 ± 0.54
Tpx2	3.38 ± 0.28**	1.16 ± 0.28
Gadd45a	2.76 ± 0.80**	1.60 ± 0.62
Cdk1	2.69 ± 0.61**	1.64 ± 0.44*
Ccnb1	2.28 ± 0.59**	1.57 ± 0.50
Aurkb	2.75 ± 0.65**	1.34 ± 0.21
Aurka	2.85 ± 0.42**	1.27 ± 0.27

Aurk aurora kinase, *Ccn* cyclin, *Cdk* cyclin-dependent kinase, *Cdkn* cyclin-dependent kinase inhibitor, *Cdr2* cerebellar degeneration-related 2, *Gadd45a* growth arrest and DNA-damage-inducible, alpha, *Tpx2* microtubule-associated, homolog (*Xenopus laevis*), *Wee1* wee 1 homolog (*S. pombe*)

* $P < 0.05$ versus untreated controls

** $P < 0.01$ versus untreated controls

[a] Numbers of animals examined were 6 in each group

[b] Mean ± SD

two-stage thyroid carcinogenesis model, suggestive of cell proliferation activity (Ago et al. 2010). We also found an increased number of cells positive for p-Histone H3 and its interaction partner HP1α, acting at the early M phase (Hirota et al. 2005), after treatment with hepatocarcinogens that induce high cell proliferation activity. The only exception was an increase in p-Histone $H3^+$ cells after treatment with PBO without increased cell proliferation. It was previously reported that p-Histone H3 and HP1α reflect cell proliferation (De Koning et al. 2009; Aune et al. 2011). Together with our previous study results, these results suggest that carcinogens inducing high cell proliferation activity produce cell populations undergoing sustained activation of the G_2/M checkpoint, resulting in cells inappropriately exiting from the G_2/M checkpoint and undergoing M phase arrest.

Like nuclear Cdc2, p-Histone H3 and HP1α, Aurora B and its interaction partner Incenp are M phase proteins. The Aurora B-Incenp complex has a function in correcting chromosome attachments (Ruchaud et al. 2007). We found that all hepatocarcinogens studied increased the number of Aurora B^+ or $Incenp^+$ cells, irrespective of the potential for inducing cell proliferation, as compared with the untreated controls and non-carcinogenic APAP and ANIT. The exception was an increase in the $Incenp^+$ population caused by APAP. Overexpression of Aurora B causes chromosomal instability in various cancer cells (Qi et al. 2007). It was reported that aberrant expression of Incenp may cause chromosomal instability or its aberrant segregation in human breast cancer cells (Nguyen and Ravid 2006). Therefore, hepatocarcinogens may cause chromosomal instability at the early stages of hepatocarcinogenesis and hepatocarcinogens that induce cell proliferation further produce cells in M phase arrest.

In the present study, $p21^{Cip1+}$ cells specifically increased after treatment with hepatocarcinogens. We did not find fluctuations in immunoreactive cellular distribution of other CDK inhibitors such as $p16^{Ink4a}$ and $p27^{Kip1}$ specific to hepatocarcinogens. Expression of $p21^{Cip1}$ is usually regulated by p53 to mediate G_1 arrest (Sherr and Roberts 1995). While the cellular distribution pattern of $p21^{Cip1}$ was similar to that of p53 after treatment with TAA, FB, and MEG, p53 did not respond to PBO in the present study. It is reported that there are a number of p53-independent mechanisms for $p21^{Cip1}$ induction (Abbas and Dutta 2009). There are a number of p53-independent $p21^{Cip1}$ inducers, such as Ndrg1 and Klf6 (Abbas and Dutta 2009; Kovacevic et al. 2011). In the present study, expression of both *Ndrg1* and *Klf6* was found to be upregulated by TAA-treatment, as shown by microarray analysis. We further found upregulation of *Klf6* by three of the four hepatocarcinogens (TAA, PBO and MEG) by real-time RT-PCR analysis as compared with untreated control animals. Therefore, it is possible that 28-day treatment with hepatocarcinogens produces cell populations arrested at the G_1 phase by $p21^{Cip1}$ via a mechanism independent of p53.

In the present study, transcript levels of *p53* and *Mdm2* significantly increased in the TAA group. $p53^+$ cells were increased after treatment with hepatocarcinogens that induced cell proliferation. It is well known that p53 induces apoptosis in response to DNA damage (Gotz and Montenarh 1995). We previously demonstrated that hepatocarcinogens that induced cell proliferation also induce apoptosis of liver cells (Taniai et al. 2012). These results suggest that hepatocarcinogens that induce cell proliferation also activate the p53 signaling cascade in response to G_1 or M phase arrest to undergo apoptosis. An increase in Mdm2 transcripts may represent facilitation of metabolic turnover of p53 (Fuchs et al. 1998).

With regard to the difference in the cellular responses on M phase proteins between PBO and other hepatocarcinogens, we think cellular responses may be parallel to the cell proliferation potential of carcinogens. It has been proposed that a non-genotoxic mode of action to induce hepatocellular apoptosis with subsequent regeneration (proliferation) is responsible for the hepatocarcinogenicity of fumonisin B_1 mycotoxin (Dragan et al. 2001). We recently found increased proliferation and apoptosis of liver cells

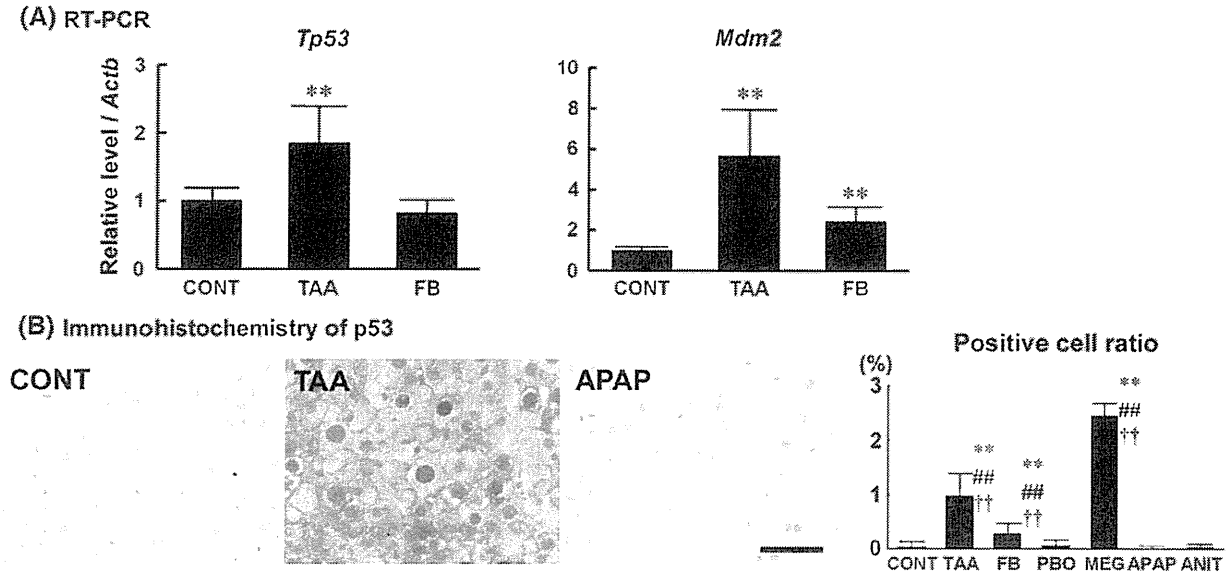

Fig. 2 Expression of p53 and downstream Mdm2 in the liver after 28-day treatment with hepatocarcinogens in rats. **a** Real-time RT-PCR analysis of p53 and Mdm2. Values are expressed as group mean fold changes over untreated controls. Values represent mean + SD *, ** $P < 0.05$, 0.01 versus untreated controls (Dunnett's or Steel's test). **b** Immunohistochemical cellular distribution of p53 in liver cells. Photomicrographs show immunoreactive cell distributions of p53 in the liver cells in representative cases of an untreated control and animals treated with TAA or APAP. The graphs show positive cell ratios (%) of liver cells per total cells counted using 10 animals in each group. Magnification: ×400 (*Bar* = 50 μm). ** $P < 0.01$ versus untreated controls (Dunnett's or Steel's test). ## $P < 0.01$ versus APAP (Dunnett's or Steel's test). †† $P < 0.01$ versus ANIT (Dunnett's or Steel's test)

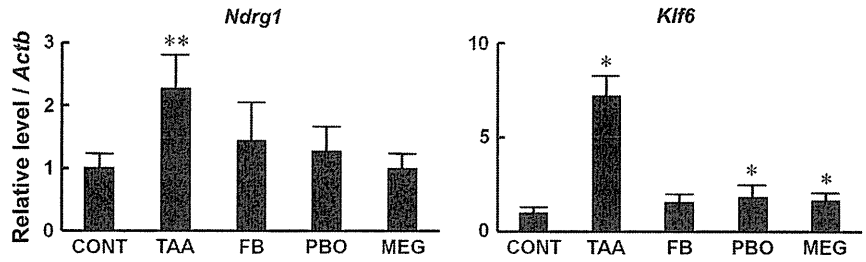

Fig. 3 Real-time RT-PCR analysis of *Ndrg1* and *Klf6*. Values are expressed as group mean fold changes over untreated controls. Numbers of animals examined were 6 in each group. Values represent mean + SD *, ** $P < 0.05$, 0.01 versus untreated controls (Dunnett's or Steel's test)

following treatment with either TAA, FB, or MEG; however, we did not find increased proliferation or apoptosis after PBO treatment (Taniai et al. 2012). It may be reasonable to hypothesize that induction of proliferation and apoptosis in carcinogenic target cells is dependent on carcinogenic potential of chemicals administered. It is well known that tumor-promoting potential of hepatocarcinogens in a rat two-stage hepatocarcinogenesis model correlates well with their hepatocarcinogenic potential (Shirai 1997). We previously reported that TAA and FB had higher tumor promoting activity than PBO (Ichimura et al. 2010), with the dose level used in the present study. MEG induces hepatocellular adenomas and carcinomas in rats after repeated oral administration at 300 mg/kg/day even at the one-year interim killing in two-year carcinogenesis test (NTP 2000). This dose is less than one-third of the dose level in the present study. Therefore, the lack of cellular responses to some M phase proteins with PBO may be a reflection of its weaker carcinogenic potential as compared with TAA, FB, and MEG.

In conclusion, hepatocarcinogens, irrespective of their cytomegaly inducing potential, increased the population of $p21^{Cip1+}$ cells, suggesting production of a cell population arrested at the G_1 phase by $p21^{Cip1}$. All hepatocarcinogens studied also increased Aurora B^+ and $Incenp^+$ cells, suggesting an increased cell population with chromosomal instability. Hepatocarcinogens that induced cell proliferation further increased the number of immunoreactive cells for p53, nuclear Cdc2, p-Histone H3, and HP1α, suggesting that cell cycle facilitation may cause M phase arrest accompanied by apoptosis. Therefore, the present study indicates that a combination of these proteins may be an early prediction marker of hepatocarcinogens in a 28-day treatment scheme in rats.

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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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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.

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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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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-67^+, Aurora B^+ or HP1α$^+$ 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.

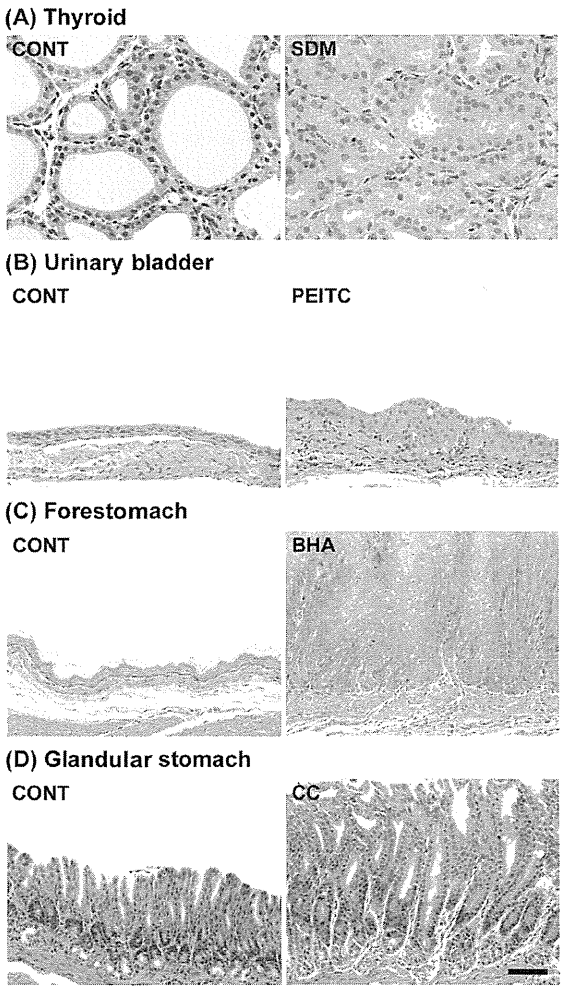

Fig. 1. Histopathological changes of the thyroid, urinary bladder, forestomach and glandular stomach in animals treated with SDM, PEITC, BHA and CC, respectively. (A) Thyroid, (B) urinary bladder, (C) forestomach and (D) glandular stomach. Bar = 50 μm (A). Bar = 100 μm (B–D).

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).

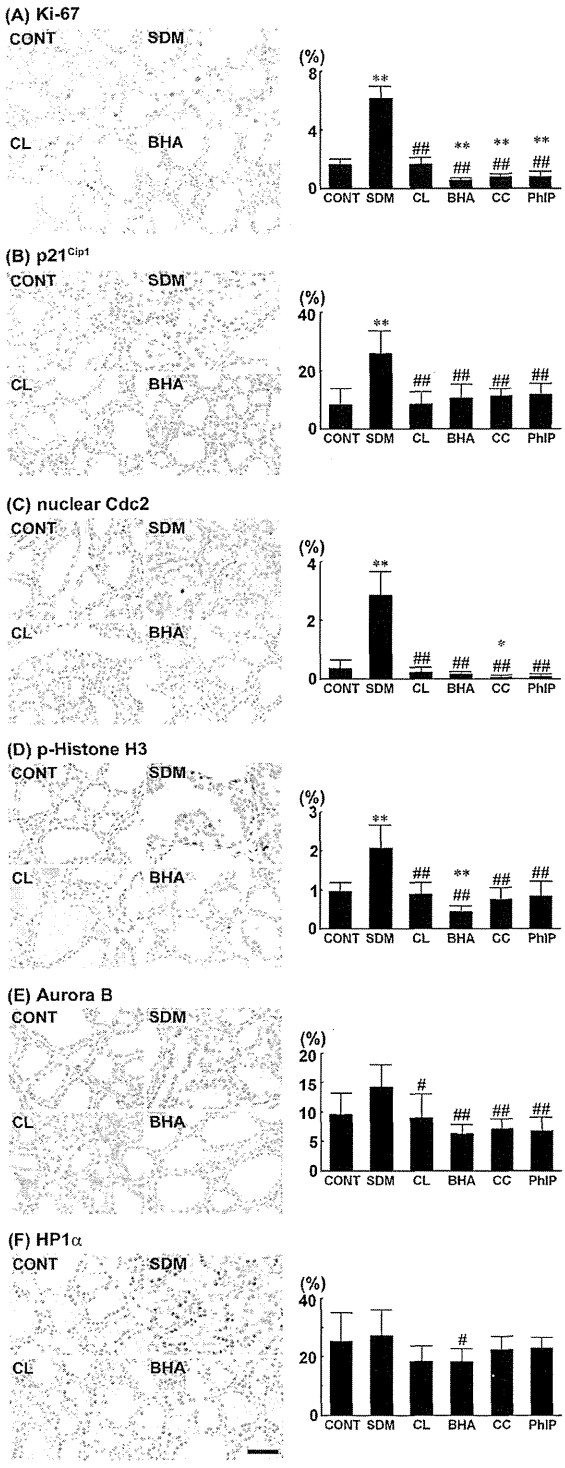

Fig. 2. Distribution of Ki-67$^+$, p21^{Cip1+}, nuclear Cdc2$^+$, p-Histone H3$^+$, Aurora B$^+$ and HP1α$^+$ cells in the thyroid. Photomicrographs show Ki-67$^+$, p21^{Cip1+}, nuclear Cdc2$^+$, p-Histone H3$^+$, Aurora B$^+$ and HP1α$^+$ cells in untreated controls and animals treated with SDM, CL 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).

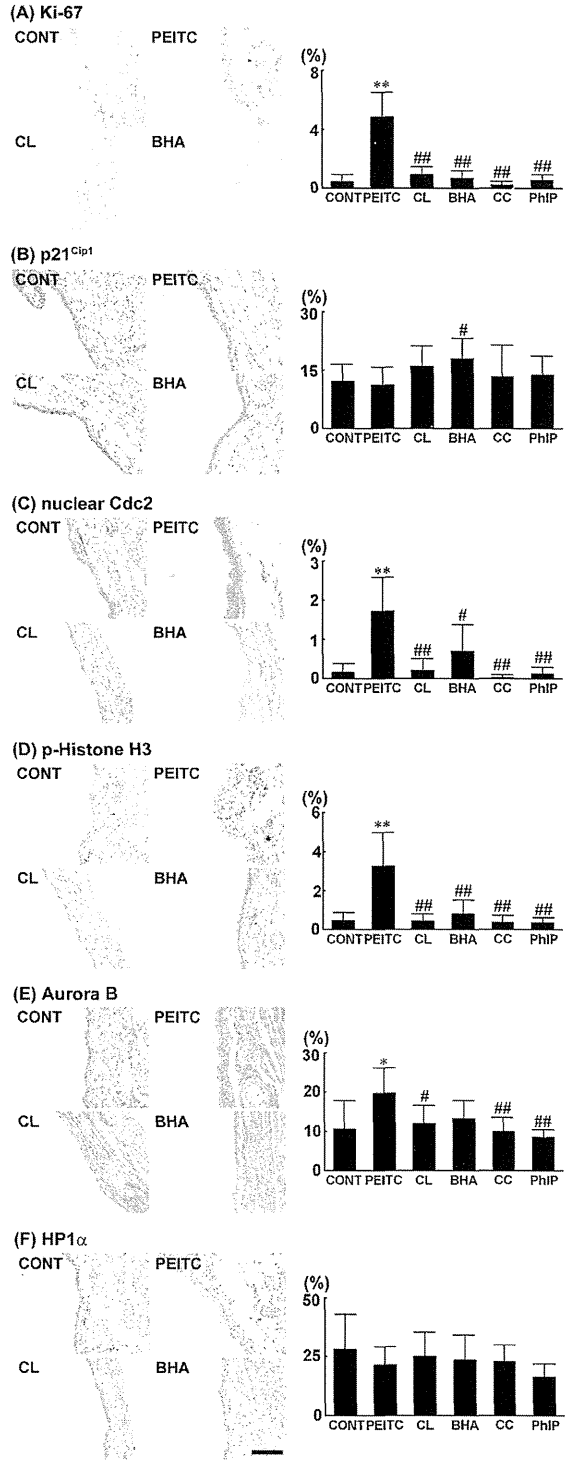

Fig. 3. Distribution of Ki-67$^+$, p21^{Cip1+}, nuclear Cdc2$^+$, p-Histone H3$^+$, Aurora B$^+$ and HP1α$^+$ cells in the urinary bladder. Photomicrographs show Ki-67$^+$, p21^{Cip1+}, nuclear Cdc2$^+$, p-Histone H3$^+$, Aurora B$^+$ and HP1α$^+$ cells in untreated controls and animals treated with 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).

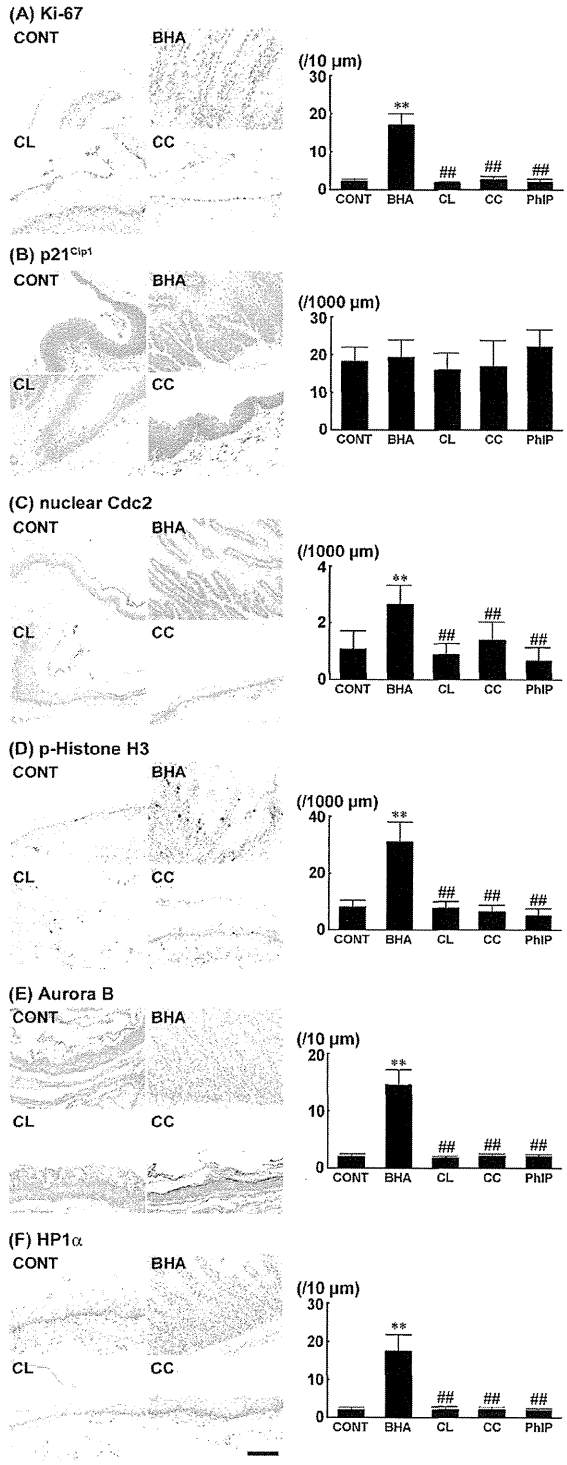

Fig. 4. Distribution of Ki-67$^+$, p21^{Cip1+}, nuclear Cdc2$^+$, p-Histone H3$^+$, Aurora B$^+$ and HP1α$^+$ cells in the forestomach. Photomicrographs show Ki-67$^+$, p21^{Cip1+}, nuclear Cdc2$^+$, p-Histone H3$^+$, Aurora B$^+$ and HP1α$^+$ cells in untreated controls and animals treated with BHA, 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).

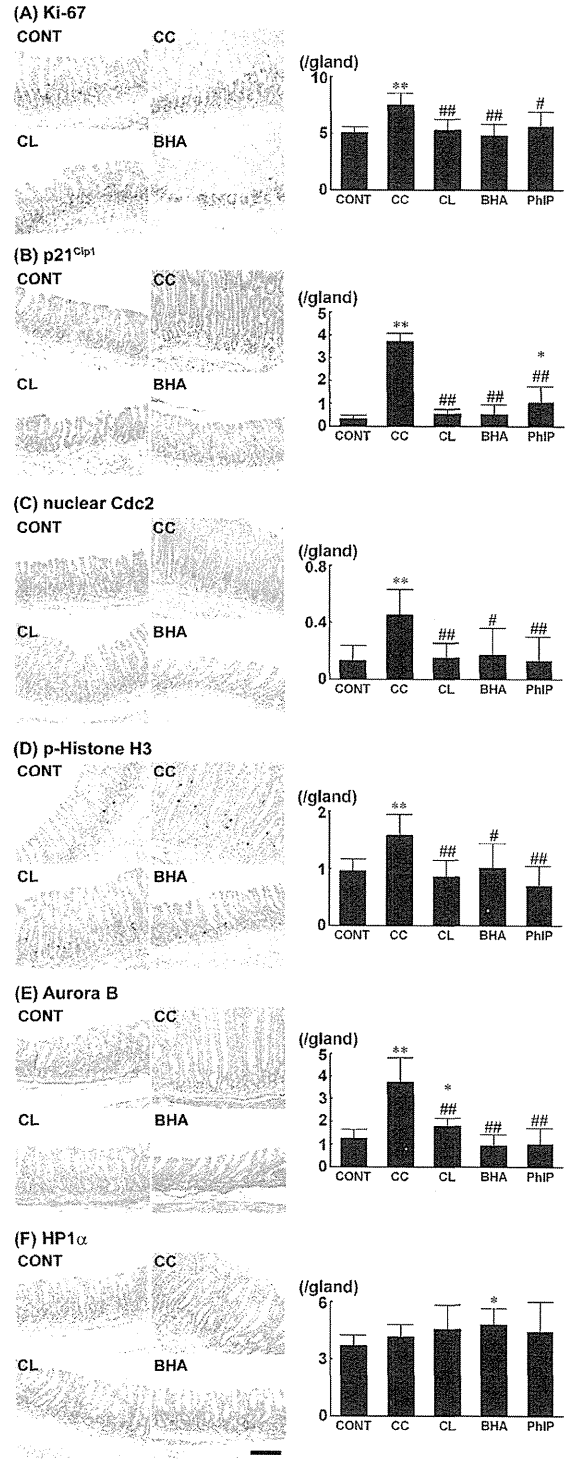

Fig. 5. Distribution of Ki-67$^+$, p21^{Cip1+}, nuclear Cdc2$^+$, p-Histone H3$^+$, Aurora B$^+$ and HP1α$^+$ cells in the glandular stomach. Photomicrographs show Ki-67$^+$, p21^{Cip1+}, nuclear Cdc2$^+$, p-Histone H3$^+$, Aurora B$^+$ and HP1α$^+$ cells in untreated controls and animals treated with 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).

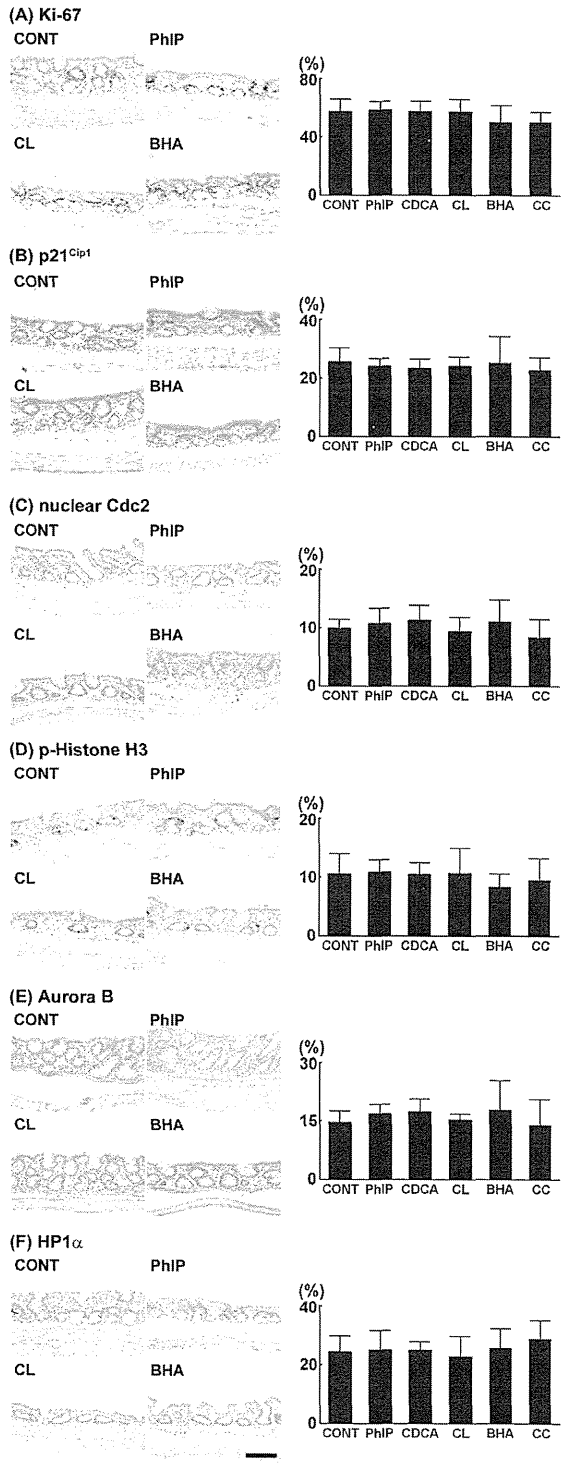

Fig. 6. Distribution of Ki-67$^+$, p21^{Cip1+}, nuclear Cdc2$^+$, p-Histone H3$^+$, Aurora B$^+$ and HP1α$^+$ cells in the colon. Photomicrographs show Ki-67$^+$, p21^{Cip1+}, nuclear Cdc2$^+$, p-Histone H3$^+$, Aurora B$^+$ and HP1α$^+$ cells in the colon in untreated controls and animals treated with 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.

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Original Article

Liver tumor promoting effect of omeprazole in rats and its possible mechanism of action

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ABSTRACT — Omeprazole (OPZ), a proton pump inhibitor, is a cytochrome P450 (CYP) 1A1/2 inducer. Some CYP1A inducers are known to have liver tumor promoting effects in rats and the ability to enhance oxidative stress. In this study, we performed a two-stage liver carcinogenesis bioassay in rats to examine the tumor promoting effect of OPZ (Experiment 1) and to clarify a possible mechanism of action (Experiment 2). In Experiment 1, male F344 rats were subjected to a two-third partial hepatectomy, and treated with 0, 138 or 276 mg/kg OPZ by oral gavage once a day for six weeks after an intraperitoneal injection of *N*-diethylnitrosamine (DEN). Liver weights significantly increased in the DEN+OPZ groups, and the number and area of glutathione *S*-transferase placental form (GST-P) positive foci significantly increased in the DEN+276 mg/kg OPZ group. In Experiment 2, the same experiment as Experiment 1 was performed, but the dosage of OPZ was 0 or 276 mg/kg. The number and area of GST-P positive foci as well as liver weights significantly increased in the DEN+276 mg/kg OPZ group. The number of proliferative cell nuclear antigen (PCNA)-positive cells also significantly increased in the same group. Real-time RT-PCR showed that the expression of AhR battery genes including *Cyp1a1*, *Cyp1a2*, *Ugt1a6* and *Nqo1*, and Nrf2 battery genes including *Gpx2, Yc2, Akr7a3, Aldh1a1 Me1 and Ggt1* were significantly upregulated in this group. However, the production of microsomal reactive oxygen species (ROS) and formation of thiobarbituric acid-reactive substances (TBARS) decreased, and 8-hydroxydeoxyguanosine (8-OHdG) content remained unchanged in this group. These results indicate that OPZ, CYP1A inducer, is a liver tumor promoter in rats, but oxidative stress is not involved in the liver tumor promoting effect of OPZ.

Key words: Omeprazole, CYP1A1/2 inducer, Tumor promotion, Rat, Liver

INTRODUCTION

Omeprazole (OPZ) is a proton pump (H^+, K^+-ATPase) inhibitor characterized by a benzimidazole structure that is widely used as a blocker of gastric acid secretion in gastric parietal cells (Ma and Lu, 2007). As a result of its efficacy, OPZ is used clinically in humans for the treatment of dyspepsia, peptic ulcer, gastro-esophageal reflux disease, and the Zollinger-Ellison syndrome (Ma and Lu, 2007). In addition, it has been reported that OPZ has an anti-proliferation effect in pancreatic cancer cell line (Udelnow *et al.*, 2011). On the other hand, oncogenicity studies have found that OPZ may induce gastric carcinoids in rats, but not in mice (Ekman *et al.*, 1985), with the finding that hyperplasia of oxyntic mucosal cells (including hyperplasia of endocrine ECL-cells and development of gastric carcinoids in rats) can be attributed to the hypergastrinemia produced as a secondary effect of inhibition of acid secretion by the large doses of OPZ used (Ekaman *et al.*, 1985). It has also been reported that OPZ can induce cytochrome P450 (CYP) 1A1/2 expression in human hepatocytes *in vivo* and *in vitro* (Diaz *et al.*, 1990).

Both the CYP1A1 and CYP1A2 enzymes can be induced by a range of chemicals in a process mediated through the aryl hydrocarbon receptor (AhR) (Ma and Lu, 2007). AhR then heterodimerizes with the AhR nuclear translocator and the heterodimer binds to the cis-ele-

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ment called xenobiotic responsive element located in the promoter region of target genes to activate their transcription (Yoshinari *et al.*, 2008). The 'classical' AhR ligands are potent activators and include the halogenated aromatic hydrocarbons like 2,3,7,8-tetrachloro-dibenzo-dioxin (TCDD) and polyaromatic hydrocarbons such as 3-methylcholanthrene and polychlorinated biphenyls (PCBs), and β-naphthoflavone (BNF) (Coe *et al.*, 2006). In contrast, several compounds known as atypical CYP1A inducers induce CYP1A without direct binding to the AhR (Lemaire *et al*, 2004), including benzimidazole compounds such as oxfendazole (OX) and indole-3-carcinol (I3C) (Coe *et al.*, 2006; Gleizes-Escala *et al.*, 1996; Lesca *et al.*, 1995). Another benzimidazole compound, OPZ is also known to induce AhR; however, the molecular mechanism underlying this process is not yet clear.

Some CYP1A inducers such as TCDD and BNF are known to have liver tumor promoting effects in rats and the ability to augment oxidative stress (Kociba *et al.*, 1987; Dewa *et al.*, 2008). It is generally accepted that microsomal electron system including CYPs and NADPH-cytochrome P450 reductase generates reactive oxygen species (ROS) via metabolism, with the subsequent formation of an oxygenated substrate and water (Poulos and Raag, 1992). Although electron transfer is normally a well-coupled process, superoxide and H_2O_2 may be released in the presence of CYP1A inducers that are poorly metabolized. In contrast, atypical CYP1A inducers such as OX and I3C also have hepatocellular tumor promoting activities (Dewa *et al.*, 2009; Shimamoto *et al.*, 2011a), and promote hepatocellular tumors via oxidative stress. In long-term carcinogenicity studies of OPZ, hepatocarcinogenicity in rats given OPZ for 2 years was not found, but treatment with 138 mg/kg OPZ induced hepatocellular hyperplastic nodules in the liver of female rats (Ekman *et al.*, 1985). In addition, oral administration of 100 mg/kg OPZ for 14 successive days to rats which had previously been treated with *N*-diethylnitrosamine (DEN) led to a significant increase in the number of gamma-glutamyltranspeptidase (Ggt) positive foci in the liver (Mereto *et al.*, 1993).

In the present study, we have performed a two-stage liver carcinogenesis bioassay in rats to examine the tumor-promoting effects of OPZ in the liver and to clarify the possible mechanism of OPZ, with a particular focus on gene expression and biochemical events of ROS generation, 8-hydroxydeoxyguanosine (8-OHdG) and thiobarbituric acid-reactive substances (TBARS) production in the liver.

MATERIALS AND METHODS

Chemicals

OPZ (CAS No. 73590-58-6) and DEN (CAS No. 55-18-5) were purchased from Wako Pure Chemical Industries (Osaka, Japan) and Tokyo Kasei Kogyo (Tokyo, Japan) with purities of 98% and 99%, respectively.

Animals

Five-week-old male F344 rats in Experiments 1 and 2 were purchased from Japan SLC, Inc. (Shizuoka, Japan), maintained in an air-conditioned room with a twelve-hour light/dark cycle (room temperature, 23°C ± 3°C; relative humidity, 55 ± 15%), and given free access to a basal diet (Oriental MF; Oriental Yeast, Tokyo, Japan) and tap water. After a one-week acclimatization period, the animals were used in the experiments. The animals received human care in accordance with the Guide for Animal Experimentation of the Tokyo University of Agriculture and Technology.

Experimental designs

Experiment 1

A medium-term liver carcinogenesis bioassay (Ito *et al.*, 2003) was performed according to the following procedure to examine the liver tumor promoting effect of OPZ: First, all rats were treated with an intraperitoneal injection of DEN at a dose of 200 mg/kg body weight, followed by treatment with 0 (DEN alone), 138 or 276 mg/kg OPZ by oral gavage once a day for six weeks starting two weeks after DEN treatment. In the previous study, since 138 mg/kg OPZ induced hepatocellular hyperplastic nodules in the liver of female rats in a long-term carcinogenicity study (Ekman *et al.*, 1985), we adopted this dose as the low dose in the present study. In our previous pilot study (Data not shown), we conducted a 2-week repeated oral dose study of OPZ at doses of 0, 37.5, 75 or 150 mg/kg body weight/day in rats and confirmed that the expression of Phase I enzyme genes such as *Cyp1a1* inducing ROS generation and phase II enzyme genes to eliminate the ROS produced such as *Yc2*, *Gpx2*, *Akr7a3* and *Nqo1* were up-regulated in the rats treated with OPZ. The expression of these genes was up-regulated in a dose-dependent manner. Therefore, we did not set the untreated and OPZ alone groups in the present study. To enhance hepatocellular proliferation, the rats were subjected to two-third partial hepatectomy at 1 week after the OPZ treatment was started. In the first two weeks, the body weight was measured once a week, and then measured every three days during the promotion period, and the OPZ dose was then determined based on these data.

At the end of the experiment, the rats were euthanized by exsanguination while under ether anesthesia and their livers were excised and weighed. The sliced liver samples were fixed in either 10% phosphate-buffered formalin for histopathological and immunohistochemical evaluations.

Experiment 2

The analysis of Experiment 1 revealed that 276 mg/kg OPZ had a liver tumor promoting activity. Therefore, we performed an additional experiment to clarify the possible mechanism of OPZ, with a particular focus on the oxidative stress. All rats were handled and treated in the same manner as in Experiment 1. In brief, two weeks after DEN treatment, the rats were administered 0 (DEN alone) or 276 mg/kg OPZ by oral gavage once a day for six weeks. In the first two weeks, the body weight was measured once a week, and then measured every three days during the promotion period, and the OPZ dose was then determined based on these data. At the end of the experiment, the rats were euthanized by exsanguination while under ether anesthesia and their livers were excised and weighed. The sliced liver samples were fixed in either 10% phosphate-buffered formalin for histopathological and immunohistochemical evaluations or were frozen in liquid nitrogen and stored at -80°C until further analysis.

Histopathology and immunohistochemistry

After formalin fixation of livers in Experiments 1 and 2, the tissues were dehydrated in graded ethanol and embedded in paraffin. Sections were then mounted onto the glass slides and stained with hematoxylin and eosin (H&E) or they were used for immunohistochemistry analysis. For immunohistochemistry, the horseradish peroxidase avidin-biotin complex method with a Vectastain Elite ABC kit (Vector Laboratories Burlingame, CA, USA) was used. Endogenous peroxidase was inhibited by incubation with freshly prepared 0.3% hydrogen peroxide with methanol for 30 min. The sections were incubated overnight with rabbit polyclonal anti-glutathione *S*-transferase placental form (GST-P) antibody (Medical & Biological Laboratories, Nagoya, Japan; 1:1000) and mouse monoclonal anti-proliferative cell nuclear antigen PCNA antibody (DAKO, Glostrup, Denmark; 1:800) at 4°C, followed by incubation with a biotinylated secondary antibody for 30 min and with avidin peroxidase conjugate for 30 min at room temperature. The sections were then developed in 0.05% 3, 3'-diaminobenzidine/hydrogen peroxide as the chromogen. For PCNA staining, the deparaffinized tissue sections were placed in an antigen-retrieval solution (0.01 M citrate buffer, pH 6.0) for 20 min in a hot bath at 60°C prior to immunohistochemical staining. After staining, the slides were lightly counterstained with hematoxylin.

The numbers and areas of GST-P positive foci (≥ 0.2 mm in diameter) and total areas of the liver sections were quantified using WinRoof software (v5.7.2; Mitani Corp., Fukui, Japan). The number of PCNA positive cells counted under 200 × magnification was expressed as a percentage of total cells counted in 20 randomly selected fields. In both analyses, cranial and caudal parts of the right lateral liver lobe were used.

cDNA microarray analysis

Total RNA of the liver obtained from Experiment 2 was extracted with an RNeasy Mini Kit (QIAGEN, Hilden, Germany), in accordance with the manufacturer's instructions. Using 10 μg of total RNA from one animal from each of the control and 276 mg/kg OPZ groups, double-stranded cDNA was synthesized with the Invitrogen Superscript Double-Stranded cDNA Synthesis kit (Invitrogen Corp., Carlsbad, CA, USA), in accordance with the manufacturer's protocol. After labeling with the Cy3, 6 μg of each of the Cy3-labeled cDNA sample were loaded onto the *Rattus norvegicus* Roche NimbleGen microarray for Gene Expression (Roche NimbleGen: Euk Expr 385K catalog Arr, 26,739 targets/microarray). Using Robust Multiple Average normalization method (Irizarry *et al.*, 2003), differentially expressed genes were analyzed. Gene information was retrieved from the National Center for Biotechnology information (http://www.ncbi.nlm.nih.gov) website.

Real-time RT-PCR analysis

Total RNA of the liver obtained from Experiment 2 was extracted with an RNeasy Mini Kit (QIAGEN), in accordance with the manufacturer's instructions. Reverse transcription was carried out with 2 μg RNA for cDNA synthesis using a Thermoscript RT-PCR System kit (Eppendorf Co., Ltd., Tokyo, Japan) in accordance with the manufacturer's protocol. Quantitative real-time RT-PCR with Power SYBR Green PCR Master Mix (Applied Biosystems Japan Ltd., Tokyo, Japan) was performed using a StepOnePlus™ Real-time PCR System (Applied Biosystems Japan Ltd.). The PCR primers (listed in Table 1) were designed using Primer Express software (Version 3.0; Applied Biosystems Japan Ltd.). The amount of target gene expression was normalized to an endogenous reference (Actin, beta) and relative to control was obtained using $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).