Original Article

Molecular mechanisms underlying ochratoxin A-induced genotoxicity: global gene expression analysis suggests induction of DNA double-strand breaks and cell cycle progression

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ABSTRACT — Ochratoxin A (OTA) is a renal carcinogen primarily affecting the S3 segment of proximal tubules in rodents. In our previous study, we reported that OTA induces reporter gene mutations, primarily deletion mutations, in the renal outer medulla (OM), specifically in the S3 segment. In the present study, to identify genes involved in OTA-induced genotoxicity, we conducted a comparative analysis of global gene expression in the renal cortex (COR) and OM of kidneys from *gpt* delta rats administered OTA at a carcinogenic dose for 4 weeks. Genes associated with DNA damage and DNA damage repair, and cell cycle regulation were site-specifically changed in the OM. Interestingly, genes that were deregulated in the OM possessed molecular functions such as DNA double-strand break (DSB) repair (*Rad18*, *Brip1*, and *Brcc3*), cell cycle progression (*Cycl1*, *Ccna2*, and *Ccnb1*), G₂/M arrest in response to DNA damage (*Chek1* and *Wee1*), and p53-associated factors (*Phlda3* and *Ccng1*). Significant increases in the mRNA levels of many of these genes were observed in the OM using real-time RT-PCR. However, genes related to oxidative stress exhibited no differences in either the number or function of altered genes in both the OM and COR. These results suggested that OTA induced DSB and cell cycle progression at the target site. These events other than oxidative stress could trigger genotoxicity leading to OTA-induced renal tumorigenicity.

Key words: Apoptosis, DNA damage, Karyomegaly, Microarray, Mycotoxin

INTRODUCTION

Ochratoxin A (OTA) is a mycotoxin produced by fungal species and is often found in food as a contaminant (EFSA, 2006). OTA is a well-known nephrotoxicant and renal carcinogen in rodents (NTP, 1989) and may be responsible for Balkan endemic nephropathy and urinary

tract tumors in humans (JECFA, 2001). Humans are still exposed to OTA in various foods (EFSA, 2006), and currently, there is no way to prevent exposure to this contaminant. However, the mechanism underlying OTA-induced renal tumorigenesis, especially the possible participation of genotoxic mechanisms, has been the subject of controversy since conventional *in vitro* and *in vivo* mutagenicity

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tests for OTA have not demonstrated consistent outcomes (JECFA, 2008).

It has been suggested that OTA behaves according to characteristic kinetics in the kidneys, possibly due to the existence of specific transporters which cause localization toxicity at the S3 segment in the renal proximal tubules (Pfohl-Leszkowicz and Manderville, 2007; Dahlmann *et al.*, 1998). In addition, OTA has been also reported to cause renal tumors at the same site (NTP, 1989; Bendele *et al.*, 1985; Boorman *et al.*, 1992). In our previous study, we observed nonuniform distribution of OTA in the kidney and therefore conducted site-specific *in vivo* mutation analysis using *gpt* delta rats, in which reporter gene mutation assays were performed separately in the renal cortex (COR) and outer medulla (OM). As a result, significant increases in the mutant frequency (MF) of the *red/gam* gene (*Spi*⁻) were observed in the OM (primarily in the S3 segment), but not in the COR, suggesting the involvement of genotoxic mechanisms in OTA-induced renal carcinogenesis (Hibi *et al.*, 2011).

Based on the positive *Spi*⁻ assay (Nohmi *et al.*, 2000, 2005), it is possible that OTA can induce deletion mutations at the carcinogenic target site. In addition to the fact that OTA-modified bases lead to the formation of apurinic sites (Cavin *et al.*, 2007), the positive outcome in the alkaline comet assay allowed us to speculate that OTA induces DNA strand breaks followed by deletion mutations (Zeljezić *et al.*, 2006). Otherwise, although there were no changes in 8-hydroxydeoxyguanine (8-OHdG) levels in kidney DNA extracted from the OM in the above-mentioned study, several lines of evidence, such as the generation of reactive oxygen species (ROS) (Schaaf *et al.*, 2002), the inhibition of Nrf-2 activity (Cavin *et al.*, 2007), and the positive results in formamidopyrimidine-DNA glycosylase (Fpg)-modified comet assays (Kamp *et al.*, 2005), indicate the possibility that oxidative DNA damage triggers these mutations. In any event, the precise mechanisms facilitating OTA-induced gene mutations *in vivo* remain uncertain (JECFA, 2008). Microarray analysis of global gene expression is a powerful tool to identify candidate genes associated with various pathogenic conditions. Marin-Kuan *et al.* (2006) reported the comprehensive analysis of gene expression in the kidneys and liver of rats treated with OTA, demonstrating the alteration of cellular homeostasis, including inhibition of anti-oxidative defense. However, it is highly probable that site-specific analysis of gene alterations may be necessary to clarify the molecular processes involved in OTA-induced DNA damage and consequent gene mutations due to the characteristic distribution of OTA in the kidneys.

In the present study, to investigate the detailed mech-

anisms involved in OTA-induced carcinogenesis, a comparison of changes in gene expression between the COR and OM was performed in the kidneys of rats treated with OTA at a carcinogenic dose for 4 weeks. In particular, we focused on the genes associated with DNA damage and DNA damage repair, oxidative stress, the cell cycle, and apoptosis, and some of these changes were also confirmed by real-time RT-PCR methods.

MATERIALS AND METHODS

Experimental animals and housing conditions

The protocol for this study was approved by the Animal Care and Utilization Committee of the National Institute of Health Sciences. Specific pathogen-free, five-week-old male F344/NSlc-Tg (*gpt* delta) rats carrying about 5 tandem copies of the transgene lambda EG10 per haploid genome were obtained from Japan SLC (Shizuoka, Japan) and acclimated for 1 week prior to testing. Animals were housed in a room with a barrier system and maintained under constant temperature ($23 \pm 2^\circ\text{C}$), relative humidity ($55 \pm 5\%$), air change (12 times/hr), and lighting (12-hr light-dark cycle), with free access to an Oriental CRF-1 basal diet (Oriental Yeast Co., Ltd., Tokyo, Japan) and tap water.

Test compound

OTA was extracted from a culture of *Aspergillus ochraceus* (BD-5). Fermentative production of OTA was performed based on the culture and extraction methods of Kumata *et al.* (1980), with minor modifications. Several 500-ml Erlenmeyer flasks containing 100 g polished rice were autoclaved before the addition of 20 ml sterilized water. The rice was inoculated with *A. ochraceus* spores and incubated for 2 weeks at 25°C . After incubation, 200 ml of a chloroform-acetic acid mixture (99:1) was added to the moldy rice in each flask. The extracts collected from the flasks were concentrated and precipitated with 4 l hexane on a stirrer. The precipitate was dissolved in 500 ml chloroform and subjected to chromatography using a silica gel column and mobile phases of benzene-acetic acid (100:0 to 88:12) with a linear gradient. The benzene-acetic acid (95:5 and 92.5:7.5) eluates were evaporated to dryness. Benzene was added to the extract, and the solution was heated, followed by gentle cooling, for crystallization of OTA. The purification of crystals was confirmed by high-performance liquid chromatography (HPLC). The purity of OTA was estimated to be greater than 95% from the area percentage of the chromatogram (data not shown).

Animal treatment

Groups of 5 male *gpt* delta rats were fed 5 ppm OTA or a basal diet without supplement for 4 weeks. The dietary dose level of 5 ppm was selected based on the carcinogenic dose reported in a 2-year carcinogenicity study of OTA in rats (Mantle *et al.*, 2005). At necropsy, as previously described, the harvested kidneys were divided into the cortex (COR), outer medulla (OM), and inner medulla (Hibi *et al.*, 2011). The CORs and OM were stored at -80°C for cDNA microarray and real-time RT-PCR.

RNA isolation

The CORs and OM from all animals were soaked overnight in RNAlater-ICE (Applied Biosystems/Ambion, Austin, TX, USA) at -20°C, and then total RNA was isolated using QIAGEN RNeasy Mini Kits (QIAGEN GmbH, Hilden, Germany) following the standard Mini Protocol for animal tissues. Briefly, COR and OM sections (15-20 mg each) were placed in 600 µl of QIAGEN RLT buffer and homogenized. Homogenates were centrifuged to remove particulate debris, and debris-free lysates were mixed with equal volumes of 70% ethanol and loaded on RNeasy spin columns. The samples were then washed 3 times on the column, and RNA was extracted in 30 µl RNase-free water. Spectrophotometric determination of the absorbance at 260 and 280 nm was made, and these measurements were used to determine the purity, concentration (ng/µl), and total yield (ng) of RNA samples. In addition, assessment of RNA quality and measurement of the 28S to 18S ratio was performed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA).

cDNA microarray

In the 3 male rats in the group, purified total RNA (100 ng) from each site was labeled with Cyanine-3 dye using the Low Input Quick Amp Labeling Kit (Agilent Technologies). RNA concentration and dye incorporation were measured using a UV-VIS spectrophotometer (Nanodrop ND-1000, NanoDrop Technologies, Wilmington, DE, USA). RNA quality was assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies). Fluorescently labeled cRNA was hybridized to 4 × 44K whole rat genome microarray gene expression chips (Agilent Technologies). Hybridization and wash processes were performed following the manufacturer's protocol (Agilent Technologies), and hybridized microarray chips were scanned using an Agilent Microarray Scanner (Model G2565BA, Agilent Technologies). Feature Extraction software (Agilent Technologies) was employed for the imaging analysis and data extraction processes. Nor-

malization of gene expression data and filtering probe sets by expression levels, flags, and errors were performed. Differences in COR and OM gene expression between treated and control rats were analyzed by analysis of variance (t-test, cut-off value: $P < 0.05$, multiple testing correction: Benjamini hochburg FDR). For the complete data at each site, a gene ontology approach was used, and DNA damage and DNA damage repair, oxidative stress, the cell cycle, apoptosis, and related genes were extracted.

Quantitative real-time RT-PCR for mRNA expression

Several key genes, each of which represent a category of genes found to be modulated by OTA, were selected to validate the microarray data. In all animals, cDNA copies of total RNA from each site were obtained using a High Capacity cDNA Reverse Transcription kit (Applied Biosystems Japan Ltd.). All PCR reactions were performed with primers for rat representative genes in the microarray data and TaqMan Rodent GAPDH Control Reagents as an endogenous reference in the Applied Biosystems 7900HT FAST Real-Time PCR Systems using the TaqMan Fast Universal PCR Master Mix and TaqMan Gene Expression Assays (Applied Biosystems Japan Ltd.). Expression levels of target genes were calculated by the relative standard curve method and were determined as ratios to GAPDH levels. Data were presented as fold-change values of OTA-treated samples relative to controls at each site. Data were analyzed statistically as follows. Variance in the data was checked for homogeneity with the F-test. When the data were homogeneous, a Student's t-test was applied. In heterogeneous cases, Welch's t-test was used instead.

RESULTS

Global gene expression analysis in the COR and OM

Under the cut-off condition of $P < 0.05$ and at least 1.5-fold change values, the expression of 1,796 genes in the COR (upregulation: 523, downregulation: 1,273) and 1726 genes in the OM (upregulation: 716, downregulation: 1010) were differentially modulated by OTA treatment. A total of 999 genes were deregulated in the COR only (upregulation: 346, downregulation: 653), while 929 genes were deregulated in the OM only (upregulation: 537, downregulation: 392). A total of 797 common genes were altered in both sites. For the genes that were up- or downregulated by OTA, a gene ontology approach was conducted, and DNA damage-, DNA damage repair-, oxidative stress-, cell cycle-, and apoptosis-related genes

Fig. 1. The number of genes modulated by OTA in the COR and OM. The number of genes categorized into DNA damage and DNA damage repair, oxidative stress, the cell cycle, and apoptosis in the COR and OM (a). The number of genes up- or downregulated in the COR only, the OM only, and both the COR and OM in DNA damage and DNA damage repair (b), oxidative stress (c), the cell cycle (d), and apoptosis (e).

were extracted (Fig. 1a). A comparison of gene expression changes between the COR and OM in response to OTA was performed, and alterations in these genes are summarized according to molecular function.

DNA damage- and DNA damage repair-related genes and their molecular functions

Twenty-five genes, which were altered only in the OM, were differentially modulated by OTA treatment, and many of these genes were upregulated (Fig. 1b and Table 1). In contrast, 11 genes were deregulated in the COR only, and many of these were downregulated (Fig. 1b and Supplementary Table 2). Nine genes were altered in both the COR and OM (Supplementary Table 3). In genes altered only in the OM, molecular functions were mainly classified as DNA double-strand break repair (DSBR), translesion synthesis (TLS), and p53-associated factors (Fig. 2a and Table 1). In order to confirm these data, representative genes altered only in the OM were analyzed by quantitative real-time PCR (Fig. 3). Most of the DNA DSBR related-genes, such as *Chek1*, *Rad18*, *Brip1*, and *Brcc3*, were deregulated at both sites. However, the degree

of change in gene expression was higher in the OM than in the COR. p53-associated factors (*Phlda3* and *Ccng1*) were upregulated only in the OM, not in the COR. TLS-related genes (*Rev1* and *Pcna*) were not changed by OTA treatment at both sites.

Oxidative stress-related genes and their molecular functions

Nine genes in the OM (Fig. 1c and Table 1) and 15 genes in the COR (Fig. 1c and Supplementary Table 2) were differentially modulated by OTA treatment. In total, 26 genes were altered in both the COR and OM (Supplementary Table 3). In genes altered only in the OM, OTA-mediated functions included superoxide metabolic processes (*Cyba*, *Cybb*, and *Sod1*), glutathione metabolic processes (*Gsta4*, *Gstt3*, *Gstk1*, and *Gpx8*), and oxidative stress processes (*Hmox1*) (Fig. 2b and Table 1). Also, genes having the same function, such as *Noxa1*, *Gsta2*, *Gstm1*, and *Gpx1* were downregulated in the COR (Supplementary Table 2).

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Table 1. Genes deregulated by OTA in the OM, as identified using global gene expression analysis

Function	Genbank Accession No.	EntrezGene ID	Gene Symbol	Gene Name	Fold change	Regulation
DNA damage and DNA damage repair						
DNA double-strand break repair	NM_080400	140583	<i>Chkl</i>	CHK1 checkpoint homolog (S. pombe)	2.69	up
	NM_001107198	305000	<i>Exo1</i>	exonuclease 1	1.50	up
	NM_001077673	362412	<i>Rad18</i>	RAD18 homolog (S. cerevisiae)	2.39	up
	NM_001108213	316344	<i>Rev1</i>	REV1 homolog (S. cerevisiae)	1.65	up
	NM_001173382	500247	<i>Ap1f</i>	apratxin and PNKP like factor	1.56	up
	NM_001109291	500987	<i>H2afx</i>	H2A histone family, member X	1.54	up
	XM_340869	360588	<i>Brip1</i>	BRCA1 interacting protein C-terminal helicase 1	2.19	up
	NM_012992	25498	<i>Npm1</i>	nucleophosmin (nucleolar phosphoprotein B23, numatrin)	1.50	up
	NM_001127300	316794	<i>Brc3</i>	BRCA1/BRC2-containing complex, subunit 3	1.68	down
Translesion synthesis	NM_022381	25737	<i>Pena</i>	proliferating cell nuclear antigen	1.94	up
	NM_001077673	362412	<i>Rad18</i>	RAD18 homolog (S. cerevisiae)	2.39	up
	NM_001108213	316344	<i>Rev1</i>	REV1 homolog (S. cerevisiae)	1.65	up
p53-associated factors	NM_033099	64576	<i>Pprv</i>	protein tyrosine phosphatase, receptor type, V	3.44	up
	NM_001012206	363989	<i>Phlda3</i>	pleckstrin homology-like domain, family A, member 3	2.90	up
	NM_001012463	373541	<i>Taf9</i>	TAF9 RNA polymerase II, TATA box binding protein (TBP)-associated factor	1.68	up
	NM_022933	65027	<i>Chd8</i>	chromodomain helicase DNA binding protein 8	1.88	up
	NM_012992	25498	<i>Npm1</i>	nucleophosmin (nucleolar phosphoprotein B23, numatrin)	1.50	up
	NM_012923	25405	<i>Ccng1</i>	cyclin G1	1.65	up
	NM_031051	81683	<i>Mif</i>	macrophage migration inhibitory factor	2.09	down
DNA methylation	NM_001007618	289508	<i>Rchy1</i>	ring finger and CHY zinc finger domain containing 1	2.26	down
	NM_022548	64394	<i>Znat3</i>	zinc finger, matrix type 3	1.83	up
	NM_001107343	306819	<i>Aof1</i>	amine oxidase (flavin containing) domain 1	2.59	down
	NM_001107276	306011	<i>Piw12</i>	piwi-like 2 (Drosophila)	2.11	up
DNA dealkylation	NM_012861	25332	<i>Mgmt</i>	O-6-methylguanine-DNA methyltransferase	1.60	up
Positive regulation of DNA repair	NM_012831	25301	<i>Cebpg</i>	CCAAT/enhancer binding protein (C/EBP), gamma	1.59	up
Response to DNA damage stimulus	NM_001107704	310707	<i>Chd11</i>	chromodomain helicase DNA binding protein 1-like	5.03	down
	NM_001107084	303836	<i>Bcl6</i>	B-cell CLL/lymphoma 6	1.90	down
Oxidative stress						
Superoxide metabolic processes	NM_023965	66021	<i>Cybb</i>	cytochrome b-245, beta polypeptide	1.66	up
	NM_024160	79129	<i>Cyba</i>	cytochrome b-245, alpha polypeptide	2.08	up
	NM_017050	24786	<i>Sod1</i>	superoxide dismutase 1, soluble	1.76	down
Glutathione metabolic processes	NM_001106840	300850	<i>Gsta4</i>	glutathione S-transferase alpha 4	1.58	down
	NM_001137643	499422	<i>Gstt3</i>	glutathione S-transferase, theta 3	4.48	down
	NM_181371	297029	<i>Gstk1</i>	glutathione S-transferase kappa 1	1.56	down
	NM_001106411	294744	<i>Gpx8</i>	glutathione peroxidase 8	1.71	up
Response to oxidative stress	NM_012580	24451	<i>Hmox1</i>	heme oxygenase (decycling) 1	2.70	up
	NM_012935	25420	<i>Cryab</i>	crystallin, alpha B	1.76	down
Cell cycle regulation						
Regulation of G ₁ phase	NM_031131	81809	<i>Tgfb2</i>	transforming growth factor, beta 2	3.09	up
	NM_001077435	363498	<i>Bex2</i>	brain expressed X-linked 2	1.78	up
Regulation of G ₁ /S phase	NM_199501	362817	<i>Cdk2</i>	cyclin dependent kinase 2	1.52	up
	NM_001100821	25729	<i>Ccncl</i>	cyclin E1	2.68	up
	NM_053702	114494	<i>Ccnad2</i>	cyclin A2	4.93	up

Table 1. (Continued)

Function	Genbank Accession No.	EntrezGene ID	Gene Symbol	Gene Name	Fold change	Regulation
Regulation of G ₂ /M phase	NM_001108976	366468	<i>Lsm10</i>	LSM10, U7 small nuclear RNA associated	1.81	down
	NM_001042354	24245	<i>Camk2b</i>	calcium/calmodulin-dependent protein kinase II beta	2.35	up
	NM_017128	29200	<i>Inhba</i>	inhibin beta-A	3.08	up
	NM_175582	291023	<i>Id4</i>	inhibitor of DNA binding 4	1.86	down
	NM_001098241	293621	<i>Hras</i>	Harvey rat sarcoma virus oncogene	1.65	up
	NM_012923	25405	<i>Ccng1</i>	cyclin G1	1.65	up
	NM_080400	140583	<i>Chek1</i>	CHK1 checkpoint homolog (S. pombe)	2.69	up
	NM_053702	114494	<i>Ccna2</i>	cyclin A2	4.93	up
	NM_001012742	308937	<i>Wee1</i>	wee 1 homolog (S. pombe)	1.65	up
	NM_001011893	287606	<i>Sept4</i>	septin 4	1.98	down
Regulation of M phase	NM_001106134	291441	<i>Skal</i>	spindle and kinetochore associated complex subunit 1	2.54	up
	NM_001009624	287598	<i>Skat2</i>	spindle and kinetochore associated complex subunit 2	1.56	up
	NM_001004415	309025	<i>Tacc2</i>	transforming, acidic coiled-coil containing protein 2	2.37	up
	NM_001012028	304951	<i>Nuf2</i>	NUF2, NDC80 kinetochore complex component, homolog (S. cerevisiae)	2.02	up
	NM_001106542	296368	<i>Ube2c</i>	ubiquitin-conjugating enzyme E2C	2.57	up
	NM_173102	29214	<i>Tubb5</i>	tubulin, beta 5	2.56	up
	NM_017100	25515	<i>Plk1</i>	polo-like kinase 1 (Drosophila)	2.59	up
	NM_171991	25203	<i>Ccnb1</i>	cyclin B1	1.53	up
	NM_001107276	306011	<i>Piwil2</i>	piwi-like 2 (Drosophila)	2.11	up
	NM_001034138	303037	<i>Ccdc99</i>	coiled-coil domain containing 99	1.59	up
Centrosome cycle	NM_012992	25498	<i>Npm1</i>	nucleophosmin (nucleolar phosphoprotein B23, numatrin)	1.50	up
Telomere maintenance	NM_001013143	307861	<i>Tert2ip</i>	telomeric repeat binding factor 2, interacting protein	1.74	up
Regulation of ubiquitin-protein ligase activity	NM_057099	29666	<i>Psm66</i>	proteasome (prosome, macropain) subunit, beta type 6	1.50	down
Apoptosis regulation						
Regulation of apoptosis related to BCL-2 family proteins	NM_053812	116502	<i>Bak1</i>	BCL2-antagonist/killer 1	1.67	up
	NM_053704	114496	<i>Bitk</i>	BCL2-interacting killer (apoptosis-inducing)	2.31	up
	NM_001077435	363498	<i>Bex2</i>	brain expressed X-linked 2	1.78	up
DNA fragmentation during apoptosis	NM_053907	116687	<i>Dnase1l3</i>	deoxyribonuclease 1-like 3	1.96	up
	NM_017050	24786	<i>Sod1</i>	superoxide dismutase 1, soluble	1.76	down
Apoptosis regulation-mediated p53 response	NM_001012206	363989	<i>Phlda3</i>	pleckstrin homology-like domain, family A, member 3	2.90	up
	NM_022548	64394	<i>Znat3</i>	zinc finger, matrin type 3	1.83	up
	NM_012992	25498	<i>Npm1</i>	nucleophosmin (nucleolar phosphoprotein B23, numatrin)	1.50	up
	NM_022933	65027	<i>Chd8</i>	chromodomain helicase DNA binding protein 8	1.88	up
	NM_012935	25420	<i>Cryab</i>	crystallin, alpha B	1.76	down
Regulation of caspase activity	NM_012923	25405	<i>Ccng1</i>	cyclin G1	1.65	up
	NM_013057	25584	<i>F3</i>	coagulation factor III (thromboplastin, tissue factor)	1.76	up
	NM_012861	25332	<i>Mgmt</i>	O-6-methylguanine-DNA methyltransferase	1.60	up
	NM_001013209	362293	<i>Dnajb6</i>	DnaJ (Hsp40) homolog, subfamily B, member 6	1.55	up
Positive regulation of anti-apoptotic responses	NM_012715	25026	<i>Adm</i>	adrenomedullin	2.18	up
	NM_053546	89807	<i>Angpt1</i>	angiopoietin 1	1.83	up
Negative or positive regulation of apoptosis	NM_001100480	246756	<i>Tradd</i>	TNFRSF1A-associated via death domain	1.54	down
Positive regulation of apoptosis	NM_173114	286894	<i>Parml</i>	prostate androgen-regulated mucin-like protein 1	1.64	down
	NM_022266	64032	<i>Ctgf</i>	connective tissue growth factor	2.70	down
	NM_017309	29748	<i>Ppp3r1</i>	protein phosphatase 3, regulatory subunit B, alpha isoform	1.50	up
	NM_012580	24451	<i>Hmox1</i>	heme oxygenase (decycling) 1	2.70	up

Fig. 2. Molecular functions of genes deregulated by OTA only in the OM. The number of genes up- or downregulated for each of the designated molecular functions: DNA damage and DNA damage repair (a), oxidative stress (b), the cell cycle (c), and apoptosis (d).

Cell cycle regulation-related genes and their molecular functions

In the OM, 34 genes were differentially modulated by OTA treatment, and many of these genes were upregulated (Fig. 1d and Table 1 and Supplementary Table 1). Fewer genes were altered in the COR than in the OM (Supplementary Table 2), and 14 genes were altered in

both the COR and OM (Supplementary Table 3). The molecular functions of genes altered only in the OM included progression or arrest in each cell cycle phase, such as G₁/S phase progression (*Ccne1* and *Ccna2*), G₂/M or M phase progression (*Ccna2* and *Ccnb1*), G₂/M phase arrest (*Ccng1*, *Chek1*, and *Wee1*), and M phase mitotic process (*Skal*, *Ska2*, *Plk1*, and so on; Fig. 2c and

Fig. 3. Changes in mRNA levels of genes associated with DNA damage and DNA damage repair as measured by quantitative real-time RT-PCR. Data show changes in mRNA levels in the COR or OM. Values are means $\pm$ SDs of data for 5 rats. * $P < 0.05$ and ** $P < 0.01$, significantly different from the COR in the control group. # $P < 0.05$ and ## $P < 0.01$, significantly different from the OM in the control group.

Table 1 and Supplementary Table 1). Moreover, in quantitative real-time PCR analysis, *Ccne1*, *Ccna2*, *Ccnb1*, *Ccng1*, *Chek1*, and *Wee1* were upregulated only in the OM, not in the COR (Figs. 3 and 4). These results suggested that OTA exerted abnormal regulatory effects on the cell cycle at the OTA target site.

Apoptosis regulation-related genes and their molecular functions

In the OM, 46 genes were differentially modulated by OTA treatment, and many of these genes were upregulated (Fig. 1e and Table 1 and Supplementary Table 1). In contrast, many of the 39 genes deregulated in the COR only were downregulated (Supplementary Table 2). Forty-two genes were altered in both the COR and OM

(Supplementary Table 3). In the OM, *Bak1*, *Bik*, and *Bex2*, categorized into the Bcl-2 family, and *Phlda3*, *Zmat3*, and *Chd8*, involved in the apoptosis pathway and mediating the regulation of p53, were upregulated (Fig. 2d and Table 1 and Supplementary Table 1). In the COR, *Bbc3* and *Bad*, belonging to the Bcl-2 family, were upregulated, but no changes in the expression of genes involved in the apoptosis pathway and mediating the regulation of p53 were observed (Supplementary Table 2). In quantitative real-time PCR analysis, *Bak1*, *Bik*, and *Phlda3* were upregulated in the OM, but not in the COR (Figs. 3 and 5).

DISCUSSION

Here, we have clarified the involvement of genotoxic

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Fig. 4. Changes in mRNA levels of genes associated with the cell cycle as measured by quantitative real-time RT-PCR. Data show changes in mRNA levels in the COR or OM. Values are means $\pm$ S.D.s of data for 5 rats. * $P < 0.05$, significantly different from the COR in the control group. # $P < 0.05$ and ## $P < 0.01$, significantly different from the OM in the control group.

Fig. 5. Changes in mRNA levels of genes associated with apoptosis as measured by quantitative real-time RT-PCR. Data show changes in mRNA levels in the COR or OM. Values are means $\pm$ S.D.s of data for 5 rats. # $P < 0.05$, significantly different from the OM in the control group.

mechanisms in OTA-induced renal carcinogenesis using reporter gene transgenic rats (*gpt* delta rats), in which deletion mutations may be predominantly induced by OTA. However, the mechanisms underlying OTA-induced mutation remain uncertain. In the present study, to identify promising candidate genes related to OTA-induced

genotoxicity, we compared gene expression changes between the COR and OM in response to OTA exposure using additional samples from *gpt* delta rats under the same conditions used in our previous study (Hibi *et al.*, 2011). Although a comprehensive gene analysis has already been performed (Marin-Kuan *et al.*, 2006), here

we have shown for the first time that genes associated with the DNA damage response were changed at the target site of OTA. In particular, genes encoding the several key regulators associated with DSBs and p53-related factors were altered in response to OTA treatment in the OM. Moreover, some of genes related to DSBs were also altered in the COR. However, more genes associated with DNA damage and DNA damage repair were altered in the OM than in the COR. These data suggested that DNA damage in the OM, a target site of OTA carcinogenicity, is more severe than in the COR.

DSBs are one of the most dangerous DNA damaging insults. Accordingly, protective systems, such as cell cycle arrest, induction of apoptosis, and DNA damage repair enzymatic networks, are active *in vivo*. DSBs are generally conducted by 2 different pathways, nonhomologous end joining (NHEJ) and homologous recombination (HR) (Tsukamoto and Ikeda, 1998). The present results showed that the expression of several genes associated with DSBs, such as *Rad18* (Huang *et al.*, 2009), *Brip1* (Peng *et al.*, 2006), *Brcc3* (Chen *et al.*, 2006), and *Chek1* (Huang *et al.*, 2008), all of which are involved in encoding HR repair enzymes for DSBs, fluctuated with OTA treatment. Also, changes in the mRNA expression of *Phlda3* and *Ccng1*, which are regulated by p53, were found (Kawase *et al.*, 2009; Okamoto and Beach, 1994). In light of a previous report demonstrating that OTA activated p53 in hepG2 cells (Bouaziz *et al.*, 2008), these results may imply activation of p53 at the target site. Interestingly, p53 has been reported to be involved in HR repair for DSBs via interaction with Rad51 (Buchhop *et al.*, 1997), BRCA1 (Ouchi *et al.*, 1998), and replication protein A (RPA) (Dutta *et al.*, 1993). In general, it has been thought that HR repair is an error-free repair process, inducing fewer gene mutations than other mechanisms. However, some reports have shown that in the process of HR repair for DSBs, large deletion mutations, distinguishable from short deletions of 1 or 2 bp, were caused (Gebow *et al.*, 2000; Bertrand *et al.*, 1997). The formation of DNA modifications such as Benzo[a]pyrene-dihydrodiol epoxide and UV light-induced pyrimidine dimers enhance the replication stress at replication forks and thereby induce DSBs (Bi *et al.*, 2005; Yajima *et al.*, 2009). It has been reported that OTA resulted in DNA adducts formation (Mantle *et al.*, 2010; Hadjeba-Medjdoub *et al.*, 2012; Pfohl-Leszkowicz and Manderville, 2012) and the subsequent induction of AP sites (Cavin *et al.*, 2007). Cleavage of AP sites by AP endonuclease has been considered to raise the possibility of the formation of single-strand breaks and then DSBs during the DNA replication (Caldecott, 2001). Additional function that OTA inhibited

topoisomerase II activity (Cosimi *et al.*, 2009) might contribute to the disorder of the normal DNA repair. Thus, although the mechanisms underlying OTA-induced DNA strand breaks including DSBs remain uncertain, positive results in the comet assay (Zeljezić *et al.*, 2006; Klarić *et al.*, 2010) support the hypothesis that OTA has the potential to cause DNA strand breaks.

Changes in the expression of genes associated with oxidative stress in the OM were not different from those in the COR in terms of the number of genes and the functions of individual genes. The present results found that various genes related to anti-oxidative enzymes, such as glutathione S-transferases, glutathione peroxidase, superoxide dismutase, heme oxygenase, and a component of NADPH oxidase, varied in response to OTA, consistent with a previous comprehensive analysis (Marin-Kuan *et al.*, 2006). With respect to the possible pathways involved in OTA-induced oxidative stress, several mechanisms, including chelating ferric ions (Omar *et al.*, 1990), ROS generation by redox cycling (Gillman *et al.*, 1999; Faucet-Marquis *et al.*, 2006), and the reduction of anti-oxidative defenses resulting from the inactivation of Nrf2 transcription activity (Cavin *et al.*, 2007), have been proposed. Thus, there is no doubt that OTA promotes ROS generation, and this was also supported by our current results. However, we previously showed that 8-OHdG levels (Hibi *et al.*, 2011) and lipid peroxidation (data not shown) were not changed at the target site. In addition, the present data revealed no differences in the fluctuation of anti-oxidative-related genes between the COR and OM, strongly suggesting that OTA-induced oxidative stress does not contribute to its genotoxicity.

By comparison of global gene expression and RT-PCR analyses in the present study, the upregulation of various genes related to the cell cycle was found specifically in the OM; these included the following genes: *Ccne1*, associated with the cell cycle transition and/or progression to G₁/S phase; *Ccna2*, associated with cell cycle progression to S or G₂ phase; *Ccnb1*, associated with the cell cycle transition and/or progression to G₂/M phase and inhibition of the cell cycle transition to G₁/M; *Chek1*, associated with the cell cycle checkpoint at G₂/M; and *Wee1*, associated with cell cycle arrest at G₂/M. Enhancement of cell proliferation at the S3 segment (Rached *et al.*, 2007) and inhibition of cell cycle progression due to cell cycle arrest at G₂/M phase (Cui *et al.*, 2010) following exposure to OTA have already been reported; the present data were able to explain these phenomena by molecular changes. The disruption of normal mitosis through the expression of cyclin-dependent kinase inhibitors, topoisomerase II, and survivin may be responsible for the character-

istic observation of karyomegaly following OTA treatment (Adler *et al.*, 2009; Rached *et al.*, 2006). Also, the present data enable us to hypothesize possible molecular mechanisms involved in the development of karyomegaly observed at the OM, including upregulation of *Chek1* acting at the G₂/M checkpoint, *Wee1* inducing G₂/M arrest downstream of *Chek1*, and *CcnA2*, *CcnB1*, and *CcnE1* mediating cell cycle progression. Together with a previous report demonstrating that Chk1 is activated by DNA strand breaks to induce polyploidy/aneuploidy during G₂/M arrest (Zheng *et al.*, 2011), the fact that HR repair mainly progresses during G₂/M phase (Rothkamm *et al.*, 2003) likely supports our proposal that predominant DNA lesion by OTA is DNA strand breaks.

The present data demonstrating changes in various p53-related genes, such as *Bik* and *Bak1* (belonging to the Bcl-2 family), *Phlda3*, and *Ccng1* may indicate activation of p53 in the OM, as mentioned above. Since OTA has been shown to induce apoptosis in proximal tubule cells of the S3 segment (Hibi *et al.*, 2011; Rached *et al.*, 2007), it is likely that p53-dependent apoptosis occurs in the OM. However, both the mitochondrial pathway, via the deregulation of Bcl-2 family proteins (Bouaziz *et al.*, 2011; Assaf *et al.*, 2004), and the p53-dependent pathway (Bouaziz *et al.*, 2008) have been proposed. *Bik*, a protein identified in the present study, is activated by genotoxic stimuli through p53-dependent or -independent mechanisms, consequently leading to the induction of apoptosis through the mitochondrial pathway (Chinnadurai *et al.*, 2008). *Bak* is also activated by p53, resulting in apoptosis through the mitochondrial pathway (Leu *et al.*, 2004). Thus, while our data failed to clarify the role of p53 in OTA-induced apoptosis, it is likely that mitochondrial-dependent pathways are involved.

In conclusion, the present data indicated that OTA induces strand breaks of target DNA including DSB and cell cycle progression, possibly leading to OTA-induced genotoxicity. Also, the present molecular analysis allows us to understand previously reported phenomena resulting from OTA treatment, such as observations of karyomegaly and apoptosis as consequences of OTA-induced DNA damage.

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