The Japanese Society of Toxicology

Fundamental Toxicological Sciences

URL : http://www.fundtoxicolsci.org/index_e.html

Letter

DNA damage mediated by 4,4'-methylenebis(2-chloroaniline) cannot be detected via the DNA damage marker γH2AX: A case study

Saho Kobayashi* and Hiroki Kashiwagi*

Division of Industrial Toxicology, Research Center for Chemical Information and Management, National Institute of Occupational Safety and Health, Japan, 6-21-1 Nagao, Tama-ku, 214-8585 Kawasaki, Japan

(Received December 13, 2022; Accepted December 19, 2022)

ABSTRACT — DNA damage may be induced by both intrinsic and extrinsic factors, the latter involving chemical exposure at workplaces. Upon DNA damage induction, checkpoint kinases such as ATM and ATR, phosphorylate serine 139 of the histone H2AX generating γH2AX, to initiate the damage response pathway. This allows antibodies that act against post-translational modifications, such as γH2AX, to be used for detecting genotoxicity. The aromatic amine 4,4'-methylenebis(2-chloroaniline) (MOCA), which is utilized in industry to produce polyurethane resins, exhibits genotoxicity and induces cancers in animals, including rodents and humans. DNA damage, due to MOCA-induced toxicity during its metabolism, is believed to be first step towards carcinogenesis. However, we failed to detect γH2AX induction by MOCA in cellular systems via western blotting, even when electrophoresis-based methods clearly indicated that physical DNA damage had been induced. In the present study, we utilized RNA sequencing of MOCA-treated cells and controls to elucidate factors underlying the discrepancies observed between these two analytical systems. Our results indicated that *H2AX* mRNA expression was significantly downregulated; this finding partially clarified the inefficient induction of γH2AX. Although downregulation of γH2AX in the presence of MOCA witnessed in this study was quite unexpected, we believe that this finding prompts researchers to be cautious when screening for genotoxicity using γH2AX only, due to the possibility of genotoxicity being overlooked in some cases. Thus, it appears that a combined approach may be more suitable for detecting genotoxicity.

Key words: γH2AX, DNA damage, Genotoxicity, 4,4'-methylenebis(2-chloroaniline) (MOCA), Aromatic amine

INTRODUCTION

DNA damage may be induced, not only by intrinsic factors such as cellular respiration, but also by environmental factors such as exposure to chemicals at the workplace (Poirier, 2004; Tubbs and Nussenzweig, 2017). Because DNA damage occurs as the initial step during carcinogenesis, it is crucial to evaluate the DNA damaging potential. Electrophoresis-based detection has been used as a canonical method to investigate such possibility. More recently, it was discovered that the formation of γH2AX, the phosphorylated form of the histone variant H2AX at Ser 139, occurred at an early stage in DNA-damage response following induction of DNA double strand break (DSB), one of the most harmful DNA damages (Rogakou *et al*., 1998). Upon DSB induction, DNA damage checkpoint kinases, including ATM (ataxia-telangiectasia mutated) and ATR (ATM- and Rad3-Related) kinases phosphorylate Ser139

Correspondence: Saho Kobayashi (E-mail: kobayashis@jniosh.johas.go.jp)
*These authors equally contributed to this work.

of H2AX at the damaged site and adjacent to the damaged site, thereby launching the overall DNA damage response signaling cascade (Lowndes and Toh, 2005; Bonner *et al*., 2008; Srivastava *et al*., 2009; Iacovoni *et al*., 2010; Pilié *et al*., 2019). Because various types of DNA damage are eventually converted to DSBs in the cellular processes such as DNA replication (Bonner *et al.*, 2008), γH2AX is widely used in genotoxic screening to evaluate the DNA damaging ability of the screening factors (Redon *et al*., 2011; Jiang *et al*., 2016; Qi *et al*., 2020; Toyoda *et al.*, 2022). More importantly, the sensitivity of γH2AX-based DNA damage detection tests is vastly superior to that of electrophoresis-based detection (Toyooka *et al*., 2011). This is because electrophoresis-based methods detect physical DNA damage per se, whereas detection of γH2AX determines amplified signaling associated with the phosphorylation at Ser 139 of H2AX by the DNA damage checkpoint kinases at the damaged site as well as at the sites surrounding it.

MOCA, an aromatic amine, which is used as a curing agent for polyurethane resins at the industrial level, has tested positive for mutagenicity in most Ames tests (McQueen and Williams, 1990); it is also known to induce various type of cancers in rodents (IARC, 2010a). Moreover, in 2010, the International Agency for Research on Cancer classified MOCA as a Group 1 human carcinogen, citing few cases of bladder cancer reported by epidemiological studies. It is widely accepted that DNA damage induced by DNA adducts that form during the metabolism of MOCA, has been proposed as the mechanism underlying MOCA-mediated carcinogenicity (IARC, 2010b). During the period from 2016 to 2018, the Ministry of Health, Labor and Welfare of Japan conducted a nation-wide screening at 538 workplaces with a history of handling MOCA and confirmed 17 cases of bladder cancer in the workers, including retired individuals.

Although we detected DNA damage induced by MOCA via electrophoresis-based methods, our attempts to determine γH2AX induction in the cellular system by western blot were unsuccessful. Hence, we explored the factors underlying the discrepancies between the results of these two methods and attempted to resolve this issue via RNA sequencing.

MATERIALS AND METHODS

Reagents

MOCA: 4,4'-methylenebis(2-chloroaniline), (CAS no: 101-14-4; purity: > 90%, Tokyo Chemical Industry CO., LTD, Tokyo, Japan) was dissolved in DMSO (FUJIFILM Wako, Osaka, Japan) and aliquots were stored at –80°C until needed. Hydrogen Peroxide (H_2O_2) (CAS no: 7722-84-1, 30%) was purchased from Wako Osaka, Japan. The primary antibodies, Anti-phospho-Histone H2A.X (Ser139) clone JBW301 (Millipore, Darmstadt, Germany) and anti-actin, C-11 (Santa Cruz Biotechnology, Dallus, TX, USA), as well as the secondary antibodies, goat anti-mouse IgG (H+L) peroxidase conjugated and rabbit anti-goat IgG (H+L) peroxidase conjugated, used in this study were purchased form Jackson Immuno Research Laboratories (West Baltimore Pike, PA, USA).

Cell line

Human hepatoma cells (HepG2) were purchased from JCRB (Japanese Collection of Research Bioresources Cell Bank). Cells were cultured in DMEM high glucose with L-glutamine and Phenol Red (FUJIFILM Wako) and supplemented with 10% FBS (Biosera, Nuaille, France) and 1% penicillin-streptomycin-amphotericin B (Wako, Osaka, Japan). Cells were cultured at 37°C under humid conditions in a 5% CO_2 incubator.

DNA damage detection via electrophoresis

Collected cells were embedded in small plugs with 1% low melting agar and treated with 0.5 mg/mL proteinase K for 48 hr at 50°C. The plugs were placed in wells of 0.8% agarose gel and electrophoresed at 20°C for 32 hr using a Biased Sinusoidal Field Gel Electrophoresis (BSFGE) system (ATTO corporation, Tokyo, Japan). DSBs were visualized after staining with ethidium bromide for 30 min.

Western Blot

Cells were collected and suspended in sample buffer (50 mM Tris-HCl (pH 8.8), 2.5 mM EDTA, 0.5% Triton X-100, 2.0% SDS, 62.5 mM Tris-HCl (pH 6.8), 10% Glycerol, 5% 2-Mercoptoetanol and 0.02% Bromophenol blue). Protein was separated via SDS-PAGE and electroblotted to polyvinylidene difluoride membranes (Millipore, Tullagreen, Ireland). The SNAP i.d.2.0 system (Merck KGaA, Darmstadt, Germany), a vacuum-driven technology, was used for western blotting. Briefly, membranes blocked with 0.25% milk were incubated with anti-γH2AX antibody (1:8000) or anti-Actin (1:2000) for 10 min, followed by goat anti-mouse IgG (H+L) peroxidase conjugated (1:8000) and rabbit anti-goat IgG (H+L) peroxidase conjugated secondary antibody (1:8000) for 10 min at room temperature. Protein detection was performed using ImmunoStar LD (FUJIFILM Wako) and visualized using a LAS-3000 imaging system (FUJIFILM, Tokyo, Japan).

RNA extraction, library preparation and sequencing

RNA extraction was performed using a RNeasy Plus Mini Kit (Qiagen, Hilden, Germany), and RNA integrity was analyzed via a Bioanalyzer system (Agilent, Tokyo, Japan). RNA concentration in the control and MOCA groups (measurements were performed in triplicate for each group) averaged 34 μg and 28 μg, respectively. Enrichment of mRNA was achieved via a NEBNext® Poly(A) mRNA Magnetic Isolation Module, E7490 (NEB, Ipswich, MA, USA), followed by a NEBNext® Ultra RNA Library Prep Kit for Illumina® (E7530) including reverse transcription with oligT containing dUTP, end modification of inserts (blunting, phosphorylation and adding dATP at 3' end), ligation of the adaptor sequence, and digestion of the second strand via USER enzyme to be strand specific. Illumina NovaSeq 6000 was used for RNA sequencing (Illumina, San Diego, CA, USA). The PE150 kit (150 bp × 2 paired-end reads) was utilized to obtain 4 gigabases (Gb) of data (26.7 M reads) per sample.

Bioinformatic analysis of the sequencing result

The quality of sequence reads was assessed using FastQC (Ver 0.11.7). Trimmomatic software (Ver 0.38) was used to trim low-quality bases and adapter sequences. Alignment of trimmed reads to the reference gene was performed via HISAT2 (Ver 2.1.0). The mapping rate of the reads in each gene were conducted using featureCounts (Ver 1.6.3). The raw read counts were normalized by relative log normalization and the analysis of differentially expressed gene (DEG) was conducted with DESeq2 (Ver 1.24.0). DEGs with the thresholds of log2(Fold Change) > 1 were selected and p value < 0.05 adjusted by Benjamini and Hochberg method were used to create the Volcano plot using plotly (Ver. 4.9.2.1). Gene Ontology analysis was performed using online resources (Gene Ontology Consortium, 2021).

RESULTS

Since MOCA is reportedly genotoxic, we first confirmed its genotoxicity using electrophoresis-based methodology. To this end, HepG2 cells were treated with MOCA at concentrations ranging from 10 μM to 100 μM. The treatment results in DNA breaks, particularly DSBs, which were detected via BSFGE. Positive controls were simultaneously treated with 2 mM H_2O_2, one of reactive oxidative species that can induce various types of DNA damage such as single strand breaks and DSBs. DNA breaks were observed at MOCA concentrations between 80 μM and 100 μM (Fig. 1). We also observed that the levels of DNA damage induced by MOCA were close

Fig. 1. DNA damage detection by Biased Sinusoidal Field Gel Electrophoresis (BSFGE). HepG2 cells were treated with DMSO (control) or MOCA for 4 hr; physical DNA breaks were observed via BSFGE.

to those induced by H_2O_2, confirming that MOCA was indeed genotoxic, as previously reported (IARC, 2010a). Trypan blue staining confirmed that both the control and MOCA exhibited more than 95% viability, thereby excluding the possibility that observed DNA breakage signals may have been caused by cell death (data not shown).

Next, we used the γH2AX-based method, which is superior in sensitivity than the electrophoresis-based method, to determine the concentration at which MOCA starts to induce DNA damage. To this end, we repeated the experiment under the same treatment conditions described earlier (Fig. 1). As expected, γH2AX was clearly induced by 2 mM H_2O_2, the same concentration used in BSFGE. Contrary to expectations, γH2AX was not induced by any of the MOCA concentrations tested, including 80 and 100 μM, at which DNA breaks were detected in BSFGE (Fig. 2). In addition, we obtained the same results repeatedly in γH2AX assay (data not shown). BSFGE results and our γH2AX-based results collectively suggested that γH2AX may not be effective in detecting the genotoxicity of MOCA. However, we were unable to elucidate the reason underlying this issue.

Finally, we resorted to RNA sequencing in an attempt

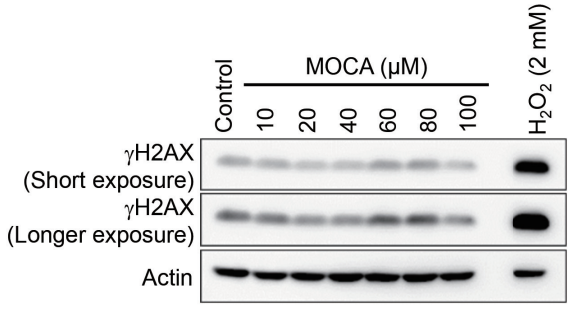

Fig. 2. γH2AX detection by western blot analysis. HepG2 cells were treated with DMSO (control) or MOCA and γH2AX and actin (loading control) were detected by western blotting following incubation for 4 hr.

to better comprehend the factors underlying the inability of MOCA to effectively upregulate γH2AX based on our results. Thus, we treated HepG2 cells with DMSO (control) or 100 μM MOCA (triplicates were performed for each group) and extracted RNA, which was sequenced after converting to cDNA.

Our results showed that mRNA such as *GDF15*, *TRIB3*, *S100P* and *C5AR1* were significantly upregulated, whereas *SLC2A3*, *TUBA1B*, *TNFRSF19* and *PCNA* were downregulated. Notably, we found *H2AX* per se in the list of highly downregulated genes (Fig. 3A). Moreover, Gene Ontology analysis of the molecular function were conducted on the highly statistically significant genes selected dotted line in A. The result showed that terms such as "response to chemical" and "response to organic substance" were evidenced in the upregulated genes, confirming the effect of MOCA treatment, whereas "chromosome organization", "Cell cycle", "DNA repair" and "regulation of DNA replication" were appeared in the downregulated genes (Fig. 3B).

DISCUSSION

Electrophoresis-based methods, such as Comet assays, were considered one of the gold standards for detecting DNA damage, until γH2AX was introduced as a DNA damage marker. Because γH2AX-based methods have come to be associated with much higher detection sensitivities, these methods have been used for genotoxic screening recently (Redon *et al*., 2011; Jiang *et al*., 2016; Qi *et al*., 2020; Toyoda and Ogawa, 2022). However, in the present study, we failed to detect γH2AX induction by MOCA, even at the concentrations where DNA breaks were observed in BSFGE (Fig. 1, Fig. 2), which could be partly attributed to the downregulation of *H2AX* mRNA detected via RNA sequencing. Even though such results were unexpected, we believe that these provide an important clue

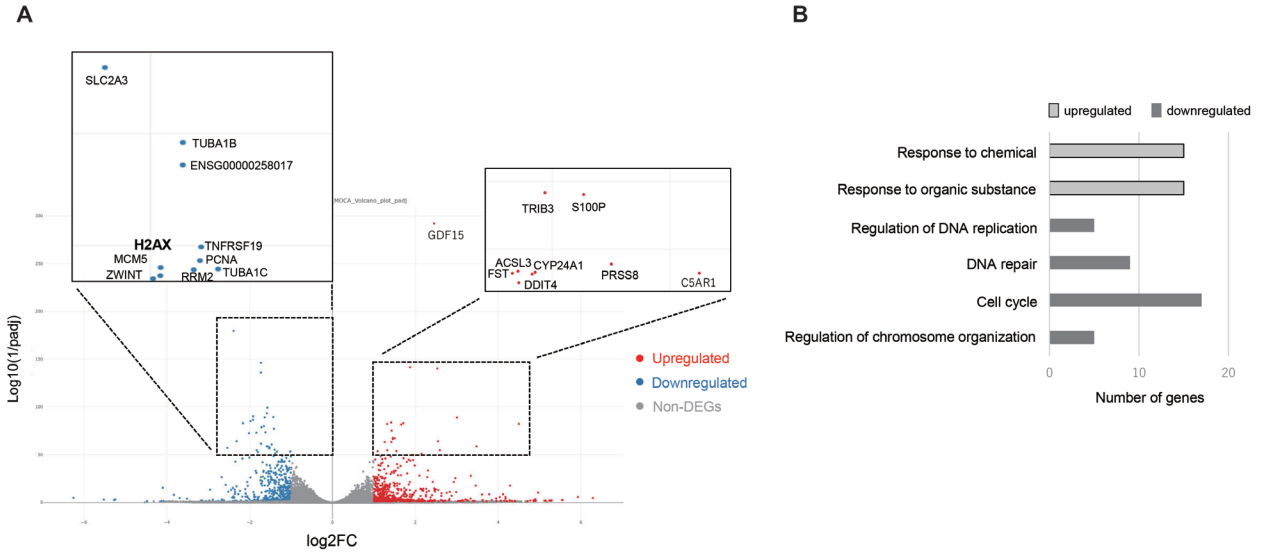

Fig. 3. Volcano plot analysis of upregulated (right) or downregulated (left) genes detected via RNA sequencing (A). The mRNA obtained from HepG2 cells treated for 24 hr for DMSO (control) or MOCA (100 μM) were sequenced. X-axis indicates the ratio of differently expressed genes (DEGs) of Control versus MOCA in log2. Y-axis indicates the statistical significance on log10 (1/p-value). Gene Ontology analysis of molecular function among the highly significant genes selected with dotted lines on A (B).

towards achieving a better understanding of MOCA-mediated cytotoxicity as well as carcinogenicity. When DSBs are induced, it is expected that γH2AX is the first to recruit DNA damage response/repair proteins to the damaged site. Thus, upon MOCA treatment, the repairing of damaged DNA may be slow and less efficient, thereby increasing the opportunity for cell death to occur. This may partially explain the cytotoxicity of MOCA being higher, compared to that of other aromatic amines, such as 4,4'-Diaminodiphenylmethane (MDA), which possess a structure similar to that of MOCA (data not shown). Moreover, as shown in Fig. 3B, there is a decrease in the mRNA levels of DNA damage response factors such as H2AX and PCNA. Hence, the probability of an imperfect repair taking place may increase, thereby enhancing the chance of a normal cell transforming into a cancerous one.

Notably, the MOCA treatment caused a slight decrease in γH2AX induction compared to control, demonstrating that MOCA may be interfering in the role played by DNA damage checkpoint kinases, such as ATM and ATR, which phosphorylate H2AX on serine 139. However, whether MOCA actually disturbs DNA damage checkpoint signaling and whether other aromatic amines are also capable of exerting a similar effect on H2AX must be addressed in a future study.

Lastly, we believe that our study highlights practical considerations for researchers engaged in screening genotoxicity using γH2AX, because our findings indicate that although γH2AX presents an attractive approach towards gaining a better understanding of DSBs as well as DNA damage in general, any screening that is based solely on γH2AX may lead to "false negatives" as pointed out previously (Toyoda and Ogawa, 2022). Therefore, it may be necessary to combine this technique with other methods or biomarkers, such as CD44, that is expressed on cancer stem cells (Yamada *et al*., 2022).

ACKNOWLEDGMENTS

RNA sequencing including RNA library preparation and bioinformatic analysis was conducted by Rhelixa Inc. (Tokyo Japan). This work partially supported by JSPS KAKENHI Grant number 21K17893. We would like to thank Editage (www.editage.com) for English language editing.

Conflict of interest---- The authors declare that there is no conflict of interest.

REFERENCES

Bonner, W.M., Redon, C.E., Dickey, J.S., Nakamura, A.J., Sedelnikova, O.A., Solier, S. and Pommier, Y. (2008): γH2AX and cancer. Nat. Rev. Cancer, **8**, 957-967.

Gene Ontology Consortium. (2021): The Gene Ontology resource: enriching a GOld mine. Nucleic Acids Res., **49**, D325-D334.

Iacovoni, J.S., Caron, P., Lassadi, I., Nicolas, E., Massip, L., Trouche, D. and Legube, G. (2010): High-resolution profiling of γH2AX around DNA double strand breaks in the mammalian genome. EMBO J., **29**, 1446-1457.

IARC (International Agency for Research on Cancer). (2010a): 4,4′-methylenebis (2-chloroaniline). IARC Monographs., **100F**, 73-82.

IARC (International Agency for Research on Cancer). (2010b): 4-aminobiphenyl. IARC Monographs., **100F**, 41-52.

Jiang, L., Wu, X., He, F., Liu, Y., Hu, X., Takeda, S. and Qing, Y. (2016): Genetic Evidence for Genotoxic Effect of Entecavir, an Anti-Hepatitis B Virus Nucleotide Analog. PLoS One, **11**, e0147440.

Lowndes, N.F. and Toh, G.W. (2005): DNA repair: the importance of phosphorylating histone H2AX. Curr. Biol., **15**, R99-R102.

McQueen, C.A. and Williams, G.M. (1990): Review of the genotoxicity and carcinogenicity of 4,4′-methylene-dianiline and 4,4′-methylene-bis-2-chloroaniline. Mutat. Res., **239**, 133-142.

Poirier, M.C. (2004): Chemical-induced DNA damage and human cancer risk. Nat. Rev. Cancer, **4**, 630-637.

Pilié, P., Chad, T., Mills, G.B. and Yap, T.A. (2019): State-of-the-art strategies for targeting the DNA damage response in cancer. Nat. Rev. Clin. Oncol., **16**, 81-104.

Qi, Y., Toyooka, T., Nie, J., Ohta, H., Koda, S. and Wang, R.S. (2020): Comparative γ-H2AX analysis for assessment of the genotoxicity of six aromatic amines implicated in bladder cancer in human urothelial cell line. Toxicol. In Vitro, **66**, 104880.

Redon, C.E., Nakamura, A.J., Gouliaeva, K., Rahman, A., Blakely, W.F. and Bonner, W.M. (2011): Q(γ-H2AX), an analysis method for partial-body radiation exposure using γ-H2AX in nonhuman primate lymphocytes. Radiat. Meas., **46**, 877-881.

Rogakou, E.P., Pilch, D.R., Orr, A.H., Ivanova, V.S. and Bonner, W.M. (1998): DNA double-stranded breaks induce histone H2AX phosphorylation on serine 139. J. Biol. Chem., **273**, 5858-5868.

Srivastava, N., Gochhait, S., de Boer, P. and Bamezai, R.N. (2009): Role of H2AX in DNA damage response and human cancers. Mutat. Res., **681**, 180-188.

Toyoda, T. and Ogawa, K. (2022): Early detection of urinary bladder carcinogens in rats by immunohistochemistry for γ-H2AX: a review from analyses of 100 chemicals. J. Toxicol. Pathol., **35**, 283-298.

Toyooka, T., Ishihama, M. and Ibuki, Y. (2011): Phosphorylation of histone H2AX is a powerful tool for detecting chemical photogenotoxicity. J. Invest. Dermatol., **131**, 1313-1321.

Tubbs, A. and Nussenzweig, A. (2017): Endogenous DNA damage as a source of genomic instability in cancer. Cell, **168**, 644-656.

Yamada, T., Toyoda, T., Matsushita, K., Akane, H., Morikawa, T., Cho, Y.M. and Ogawa, K. (2022): Persistent γ-H2AX Formation and Expression of Stem Cell Markers in N-Butyl-N-(4-Hydroxybutyl)Nitrosamine-Induced Bladder Carcinogenesis in Rats. Toxicol. Sci., **189**, 51-61.