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Downregulation of UBE2E2 in rat liver cells after hepatocarcinogen treatment facilitates cell proliferation and slowing down of DNA damage response in GST-P-expressing preneoplastic lesions

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ABSTRACT

We previously found downregulation of ubiquitin-conjugating enzyme E2E 2 (UBE2E2) in GST-P-positive $^{(+)}$ proliferative lesions produced by tumor promotion from early hepatocarcinogenesis stages in rats. Here we investigated the role of UBE2E2 downregulation in preneoplastic lesions of the liver and other target organs produced by tumor promotion in rats. Increased number of UBE2E2-related ubiquitination target proteins, phosphorylated c-MYC, KDM4A and KMT5A, was found in the UBE2E2-downregulated GST-P$^+$ foci, compared with GST-P$^+$ foci expressing UBE2E2. However, p21$^{WAF1/CIP1}$, another UBE2E2 target protein, did not increase in the positive cells. Furthermore, the numbers of PCNA$^+$ cells and γH2AX$^+$ cells were increased in UBE2E2-downregulated foci. These results suggest sustained activation of c-MYC and stabilization of KMT5A to result in c-MYC-mediated transcript upregulation and following KMT5A-mediated protein stabilization of PCNA in GST-P$^+$ foci, as well as KDM4A stabilization resulting in slowing down of DNA damage response in these lesions. Similar results were also observed in GST-P$^+$ foci produced by repeated treatment of rats with a hepatocarcinogen, thioacetamide, for 90 days. Hepatocarcinogen treatment for 28 or 90 days also increased the numbers of liver cells expressing UBE2E2-related ubiquitination target proteins, as well as PCNA$^+$ or γH2AX$^+$ cells. Conversely, UBE2E2 downregulation was lacking in PPARα agonist-induced hepatocarcinogenesis, as well as in carcinogenic processes targeting other organs, suggestive of the loss of UBE2E2-related ubiquitination limited to hepatocarcinogenesis producing GST-P$^+$ proliferative lesions. Our results suggest that repeated hepatocarcinogen treatment of rats causes stabilization of UBE2E2-related ubiquitination target proteins in liver cells to promote carcinogenesis.

1. Introduction

Evaluation of chemical carcinogenicity is crucial for assessment of chemical safety. However, standard carcinogenicity bioassays in which hundreds of rodent animals are administered test compounds over a prolonged period are time-consuming and costly. In a previous study to identify early prediction marker molecules of rat hepatocarcinogenesis, we reported that administration of carcinogens for 28 days induces expression changes in cell-cycle-related molecules resulting in cell cycle arrest in many target organs (Kimura et al., 2016; Taniai et al., 2012a; Yafune et al., 2013). Considering that cell cycle arrest is a typical feature of cellular senescence (Campisi, 2013), our previous results suggest

Abbreviations: AOM, azoxymethane; BBN, *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine; BNF, β-naphthoflavone; CF, clofibrate; DEHP, bis(2-ethylhexyl) phthalate; DEN, *N*-nitrosodiethylamine; DHPN, *N*-bis(2-hydroxypropyl)nitrosamine; FB, fenbendazole; γH2AX, phosphorylated H2A histone family, member X; GST-P, glutathione *S*-transferase placental form; KDM4A, lysine demethylase 4A; KMT5A, lysine methyltransferase 5A; MNNG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine; MP, methapyrilene hydrochloride; PB, phenobarbital; PBO, piperonyl butoxide; PCNA, proliferating cell nuclear antigen; PEITC, 2-phenylethyl isothiocyanate; PMZ, promethazine hydrochloride; PPAR, peroxisome proliferator-activated receptor; RNF8, ring finger protein 8; SDM, sulfadimethoxine sodium salt; TAA, thioacetamide; TP53BP1, tumor protein p53 binding protein 1; UBE2E2, ubiquitin-conjugating enzyme E2E 2

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that repeated exposure to carcinogenic stimuli may cause an increased number of liver cells to undergo senescence.

In our recent study, we hypothesized that epigenetic modification, such as DNA methylation, may occur in cell populations that show growth advantages during the course of carcinogen treatment. Furthermore, genes that are hypermethylated and downregulated during the course of carcinogen treatment may become early but irreversible markers of cell responses to carcinogens (Mizukami et al., 2017). In accordance with this hypothesis, we examined hypermethylated genes in the rat liver after treatment for 28 days with the hepatocarcinogen, thioacetamide (TAA), using CpG island microarrays to identify early marker molecules that direct liver cells to undergo hepatocarcinogenesis. Among the hypermethylated and downregulated genes, we identified *Ube2e2* to show increase in the number of liver cell foci downregulating immunohistochemical expression of the corresponding gene product protein, ubiquitin-conjugating enzyme E2E 2 (UBE2E2), in a subpopulation of early preneoplastic liver cell foci immunoreactive for glutathione *S*-transferase placental form (GST-P), a preneoplastic liver cell lesion marker in rats (Ito et al., 1998; Shirai, 1997). In the late stage of tumor promotion by TAA, we also found a higher incidence of UBE2E2 downregulation in GST-P-positive $^{(+)}$ adenomas and carcinomas compared with that in GST-P^{+} preneoplastic liver cell foci. Considering the role of UBE2E2 in the destabilization of cell cycle regulatory proteins (Shibata et al., 2011), these results indicate that epigenetic downregulation of *Ube2e2* gene facilitates cell proliferation after only 28 days of hepatocarcinogen treatment to contribute to tumor development.

UBE2E2 is a member of the UBE2E family of ubiquitin-conjugating enzymes that promotes ubiquitination-mediated degradation of the cell cycle regulatory molecule, p21$^{WAF1/CIP1}$, and cell proliferation-related molecules, c-MYC and lysine methyltransferase 5A (KMT5A; also known as SETD8) (Chen et al., 2016; Shibata et al., 2011). On the other hand, UBE2E2 may also mediate the degradation of lysine demethylase 4A (KDM4A), because KDM4A undergoes proteasome degradation by ring finger protein 8 (RNF8), a binding partner for UBE2E2, resulting in inhibition of binding of tumor protein p53 binding protein 1 (TP53BP1) to DNA damage sites and delay of DNA damage repair (Mallette et al., 2012). These findings suggest that UBE2E2 may have multiple functions in cell proliferation and DNA damage via ubiquitin proteasome degradation of target proteins. Among the target proteins of UBE2E2-mediated ubiquitination, p21$^{WAF1/CIP1}$ is a cyclin-dependent kinase inhibitor that plays an essential role in cell cycle arrest initiated by p53 activation in cellular senescence (Lujambio, 2016). In contrast to p21$^{WAF1/CIP1}$, both c-MYC and KMT5A are essential for cell proliferation and loss of c-MYC or KMT5A triggers cellular senescence (Tanaka et al., 2017; Wu et al., 2007). Therefore, we hypothesize that the epigenetic downregulation of *Ube2e2* in the liver of rats treated with TAA for 28 days in our previous study may contribute to cellular senescence and further molecular steps toward carcinogenesis by disruption of cellular senescence through the stabilization of p21$^{WAF1/CIP1}$, c-MYC, KMT5A and KDM4A.

In this study, we examined the relationship between downregulation of UBE2E2 and stabilization of ubiquitination target proteins for acquisition of growth advantage in GST-P^{+} preneoplastic liver cell foci in tumor promotion processes in a two-stage hepatocarcinogenesis model. We also examined whether UBE2E2 downregulation is involved in GST-P-negative $^{(-)}$ preneoplastic liver lesions produced by peroxisome proliferator-activated receptor (PPAR) α agonists and in proliferative lesions produced in the thyroid, urinary bladder, forestomach, glandular stomach and intestines in the rat carcinogenesis models. To confirm our hypothesis regarding disruption of cellular senescence to drive cells into carcinogenesis during the early stages of hepatocarcinogen treatment, we further examined the immunohistochemical distribution of UBE2E2-related ubiquitination target proteins and related molecules in the liver of rats treated with hepatocarcinogens for 28 and 90 days. Finally, we examined the relationship between downregulation of UBE2E2 and stabilization of ubiquitination target proteins in GST-P^{+} liver cell foci produced by repeated TAA treatment for 90 days without tumor initiation.

2. Materials and methods

2.1. Chemicals and animals

TAA (CAS No. 62-55-5, purity: ≥ 98%), β-naphthoflavone (BNF; CAS No. 6051-87-2, purity: ≥ 98%), phenobarbital sodium salt (PB; CAS No. 57-30-7, purity: ≥ 98%), catechol (CAS No. 120-80-9, purity: > 99%), promethazine hydrochloride (PMZ; CAS No. 58-33-3, purity: > 98%) and methylcellulose 400 were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Piperonyl butoxide (PBO; CAS No. 51-03-6, purity: ≥ 90%) was purchased from Acros Organics (Geel, Belgium), and *N*-bis(2-hydroxypropyl)nitrosamine (DHPN; CAS No. 53609-64-6) from Nacalai Tesque (Kyoto, Japan). Azoxymethane (AOM; CAS No. 25843-45-2, purity: ≥ 98%), fenbendazole (FB; CAS No. 43210-67-9, purity: ≥ 98%), sulfadimethoxine sodium salt (SDM; CAS No. 1037-50-9, purity: ≥ 98%) and methapyrilene hydrochloride (MP; CAS No. 135-23-9) were purchased from Sigma-Aldrich Japan K.K. (Tokyo, Japan). *N*-nitrosodiethylamine (DEN; CAS No. 55-18-5, purity: ≥ 98%), *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG; CAS No. 70-25-7, purity: > 95%), *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine (BBN; CAS No. 3817-11-6, purity: > 90%), 2-phenylethyl isothiocyanate (PEITC; CAS No. 2257-09-2, purity: ≥ 97%), clofibrate (CF; CAS No. 637-07-0, purity: ≥ 98%) and bis(2-ethylhexyl) phthalate (DEHP; CAS No. 117-81-7, purity: ≥ 98%) were obtained from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). In all experiments, five-week-old male F344/NSlc rats were purchased from Japan SLC, Inc. (Hamamatsu, Japan) and acclimatized to a powdered basal diet (CRF-1; Oriental Yeast Co., Ltd., Tokyo, Japan) and tap water ad libitum. They were housed in plastic cages with paper chip bedding in a barrier-maintained animal room on a 12 h light-dark cycle and conditioned at 23 ± 2 °C with a relative humidity of 55 ± 15%.

2.2. Experimental design

There were six animal experiments.

2.2.1. Experiments 1 and 2

To investigate the involvement of UBE2E2 downregulation in GST-P^{+} liver cell foci produced by tumor promotion, animals were subjected to two-stage hepatocarcinogenesis using a medium-term liver bioassay (Ito et al., 1998; Shirai, 1997). After a one-week acclimatization period, all animals were initiated with a single intraperitoneal injection of DEN (200 mg/kg body weight, dissolved in saline). Two weeks later, animals were randomly divided into five groups consisting of 12 animals per group in Experiment 1 and three groups consisting of 15 animals per group in Experiment 2. In Experiment 1, animals were provided a basal diet (DEN-alone) or a diet containing TAA at 400 ppm (DEN + TAA), MP at 1000 ppm (DEN + MP), BNF at 5000 ppm (DEN + BNF) or PBO at 15,000 ppm (DEN + PBO) for six weeks. In Experiment 2, animals were provided a basal diet (DEN-alone) or a diet containing FB at 3500 ppm (DEN + FB) or PB at 500 ppm (DEN + PB) for eight weeks. The dose levels of TAA, MP, BNF, PBO, FB and PB have shown to promote hepatocarcinogenesis in rats (Becker, 1983; Hayashi et al., 2012; Kitano et al., 1998; Muguruma et al., 2009; NTP, 2000; Shoda et al., 1999). The animals were subjected to two-thirds partial hepatectomy at week 3. At week 8 (Experiment 1) or 10 (Experiment 2), all animals were euthanized by exsanguination from the posterior vena cava and abdominal aorta under CO_2/O_2 anesthesia. Livers were immediately removed and weighed, and then liver slices (two slices per animal) were fixed in 4% (w/v) paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for two days and processed for histopathological examinations. Except for DEN + MP group, animal samples were