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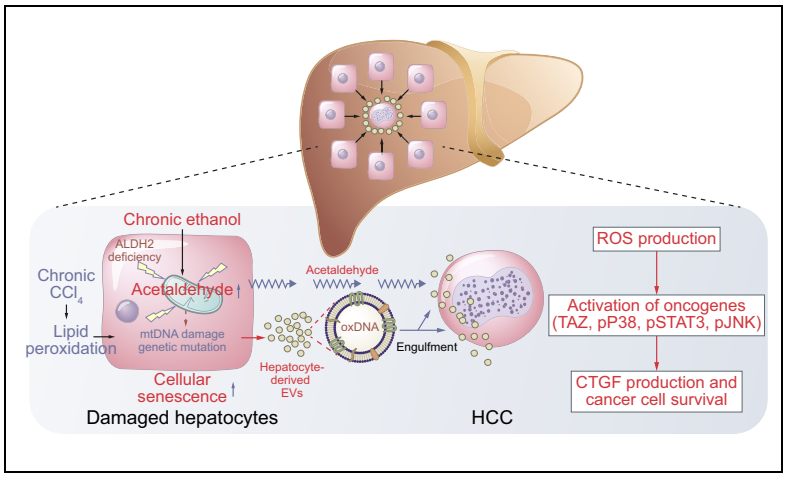
Graphical abstract



Highlights

- ALDH2 deficiency is associated with an increased risk of HCC from cirrhosis in those who drink alcohol.
- Chronic CCl_4+EtOH treatment induces greater hepatic mitochondrial DNA damage in *Aldh2*-deficient mice than WT mice.
- Oxidized mitochondrial DNA is delivered to HCC cells via hepatocyte-derived extracellular vesicles.
- Oxidized mitochondrial DNA and acetaldehyde synergistically promote ROS production and multiple oncogenic pathways.

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Lay summary

Alcoholics with an *ALDH2* polymorphism have an increased risk of digestive tract cancer development, however, the link between ALDH2 deficiency and hepatocellular carcinoma (HCC) development has not been well established. In this study, we show that ALDH2 deficiency exacerbates alcohol-associated HCC development both in patients and mouse models. Mechanistic studies revealed that after chronic alcohol exposure, *Aldh2*-deficient hepatocytes produce a large amount of harmful oxidized mitochondrial DNA via extracellular vesicles, which can be delivered into neighboring HCC cells and subsequently activate multiple oncogenic pathways, promoting HCC.

http://dx.doi.org/10.1016/j.jhep.2019.06.018
Published by Elsevier B.V. on behalf of European Association for the Study of the Liver. J. Hepatol. 2019, 71, 1000–1011

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ALDH2 deficiency promotes alcohol-associated liver cancer by activating oncogenic pathways via oxidized DNA-enriched extracellular vesicles

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Background & Aims: Excessive alcohol consumption is one of the major causes of hepatocellular carcinoma (HCC). Approximately 30–40% of the Asian population are deficient for aldehyde dehydrogenase 2 (ALDH2), a key enzyme that detoxifies the ethanol metabolite acetaldehyde. However, how ALDH2 deficiency affects alcohol-related HCC remains unclear.

Methods: *ALDH2* polymorphisms were studied in 646 patients with viral hepatitis B (HBV) infection, who did or did not drink alcohol. A new model of HCC induced by chronic carbon tetrachloride (CCl_4) and alcohol administration was developed and studied in 3 lines of *Aldh2*-deficient mice: including *Aldh2* global knockout (KO) mice, *Aldh2*1/*2* knock-in mutant mice, and liver-specific *Aldh2* KO mice.

Results: We demonstrated that ALDH2 deficiency was not associated with liver disease progression but was associated with an increased risk of HCC development in cirrhotic patients with HBV who consumed excessive alcohol. The mechanisms underlying HCC development associated with cirrhosis and alcohol consumption were studied in *Aldh2*-deficient mice. We found that all 3 lines of *Aldh2*-deficient mice were more susceptible to CCl_4 plus alcohol-associated liver fibrosis and HCC development. Furthermore, our results from *in vivo* and *in vitro* mechanistic studies revealed that after CCl_4 plus ethanol exposure, *Aldh2*-deficient hepatocytes produced a large amount of harmful oxidized mitochondrial DNA via extracellular vesicles, which were then transferred into neighboring HCC cells and together with acetaldehyde activated multiple oncogenic pathways (JNK, STAT3, BCL-2, and TAZ), thereby promoting HCC.

Conclusions: ALDH2 deficiency is associated with an increased risk of alcohol-related HCC development from fibrosis in patients and in mice. Mechanistic studies reveal a novel mechanism that *Aldh2*-deficient hepatocytes promote alcohol-associated HCC by transferring harmful oxidized mitochondrial DNA-enriched extracellular vesicles into HCC and subsequently activating multiple oncogenic pathways in HCC.

Lay summary: Alcoholics with an *ALDH2* polymorphism have an increased risk of digestive tract cancer development, however, the link between ALDH2 deficiency and hepatocellular carcinoma (HCC) development has not been well established. In this study, we show that ALDH2 deficiency exacerbates alcohol-associated HCC development both in patients and mouse models. Mechanistic studies revealed that after chronic alcohol exposure, *Aldh2*-deficient hepatocytes produce a large amount of harmful oxidized mitochondrial DNA via extracellular vesicles, which can be delivered into neighboring HCC cells and subsequently activate multiple oncogenic pathways, promoting HCC.

Introduction

Hepatocellular carcinoma (HCC) is a leading cause of cancer-related death worldwide. Chronic alcohol abuse is a major cause of HCC development; its metabolite, acetaldehyde is believed to play an important role in inducing HCC.[1] Mitochondrial aldehyde dehydrogenase (ALDH2) is a major enzyme for acetaldehyde elimination. The Glu487Lys polymorphism (also named rs671, with the glutamate corresponding to *1 allele, and lysine corresponding to *2 allele) at codon 487 in the *ALDH2* gene causes the substitution of glutamate (Glu) by lysine (Lys), which exists in approximately 40% of east Asian populations.[2] Such a polymorphism (Glu to Lys, or G to A, or *1 to *2) disrupts ALDH2 activity, causing high blood acetaldehyde concentration and "alcohol flush reactions" after alcohol consumption.[1] Alcoholics with heterozygous *ALDH2*1/*2* or homozygous *ALDH2*2/*2* polymorphism have ALDH2 deficiency and have an increased risk of developing digestive tract cancers,[3] however, the

Keywords: Aldehyde dehydrogenase 2; Ethanol; Acetaldehyde; Hepatocellular carcinoma; Cirrhosis; Extracellular vesicles; HBV; TAZ.
Received 28 February 2019; received in revised form 14 June 2019; accepted 19 June 2019; available online 4 July 2019
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association of this polymorphism with HCC development and how acetaldehyde affects HCC still remain obscure.

A case-control study of a small number of patients (78 cases) suggests that the frequency of the *ALDH2*2/*2* allele correlated with an increased risk of HCC among heavy drinkers,[4] but it is not clear whether this *ALDH2* polymorphism is associated with HCC caused by etiologies other than alcohol. In the current study, we examined 646 patients with viral hepatitis B (HBV) infection and found that ALDH2 deficiency was not associated with an increased risk of HCC development in patients with HBV, without alcohol consumption, but was a risk factor for HCC in cirrhotic patients with HBV who consumed excessive alcohol, suggesting that cirrhosis, alcohol consumption, and ALDH2 deficiency synergistically promote HCC. To model this clinical condition, we developed a mouse model of HCC induced by chronic CCl_4 administration (fibrosis) and alcohol feeding, and tested this model in 3 lines of *Aldh2*-deficient mice: including *Aldh2* global knockout (KO), *Aldh2*1/*2* knock-in, and liver-specific *Aldh2* KO ($Aldh2^{Hep-/-}$) mice. Our data revealed that all of these *Aldh2*-deficient mice were more susceptible to CCl_4 plus alcohol-associated HCC development than their wild-type (WT) counterparts.

ALDH2 deficiency is known to induce excessive acetaldehyde accumulation and oxidative stress during alcohol consumption, leading to mitochondrial DNA damage and base modifications such as oxidation of deoxyguanosine to 8-hydroxy-2′-deoxyguanosine (8-OhDG) leading to mitochondrial dysfunction.[5] Consequently, mitochondrial dysfunction-mediated DNA damage further accelerates cellular senescence, and contributes to aging-associated phenotypes and pathologies in various types of diseases and cancer including HCC.[6] Herein, we provide evidence suggesting that extracellular vesicles (EVs) can transfer this damaged DNA from hepatocytes into HCC cells, thereby promoting HCC progression, suggesting a cross-talk between damaged hepatocytes and HCC cells via the transfer of oxidized mitochondrial DNA (mtDNA)-enriched EVs that promote HCC.

EVs are well-known as a means of intercellular communication, capable of delivering proteins, miRNAs, DNAs, and glycolipids. They play important roles in the physiology and pathology of the liver.[7,8] Interestingly, EVs are also commonly detected in the tumor microenvironment and play a role in facilitating tumorigenesis by regulating angiogenesis, immunity, and metastasis.[9] In the current study, we demonstrated that after chronic CCl_4 and ethanol exposure, *Aldh2*-deficient hepatocytes produced oxidized mtDNA-enriched EVs, which were transferred into HCC cells and activated multiple oncogenic pathways that have previously been implicated in promoting HCC. These pathways include signal transducer and activator of transcription 3 (STAT3),[10,11] C-Jun N-terminal kinase (JNK),[12,13] transcriptional co-activator with PDZ-binding motif (TAZ),[14,15] and BCL-2 protein.[16,17]

Materials and methods

Human subject cohort

A total of 929 individuals were included in the study, including 102 cases with HBV-related chronic hepatitis, 264 cases with HBV-related cirrhosis, 280 cases with HBV-related HCC, and 283 healthy individuals as controls. Among patients with HCC, 116 individuals drank alcohol (male >40 g/day, female >20 g/day, drinking period >5 years), and these patients are defined as HCC with excessive alcohol consumption. The remaining patients with HCC were defined as HCC without alcohol consumption. The baseline demographic and clinical characteristics of this cohort were described in supporting materials and Table S1. *ALDH2* rs671 includes 3 genotypes, GG, AA, and GA (Table S2). The study was approved by the First Affiliated Hospital of Jilin University Institutional Review Board and the Research and Development Committee. All participants provided written informed consent.

Animals

Aldh2 knockout ($Aldh2^{-/-}$) mice on a C57BL/6N background have been described previously[18] and C57BL/6N mice were used as controls. Heterozygous *Aldh2*1/*2* knock-in mice were kindly provided by Dr. Mochly-Rosen.[19] *Aldh2*1/*2* mice were crossed with C57BL/6N mice to generate *Aldh2*1/*2* knock-in mice and WT littermate controls. The *Aldh2* floxed mice were generated by activating the *Aldh2* gene in $Aldh2^{tm1a(EUCOMM)Wtsi}$ mice by crossing of $Aldh2^{tm1a(EUCOMM)Wtsi}$ mice (kindly provided by Dr. Patel)[20] with homozygous FLPcR mice (The Jackson Laboratory), which express Flippase (flip) in germline cells. Liver-specific *Aldh2* knockout ($Aldh2^{Hep-/-}$) mice were generated via several steps of crossing *Aldh2* floxed mice with Albumin-Cre mice (The Jackson Laboratory). Mice were housed in polycarbonate cages (4 mice per cage) and maintained in a temperature and light controlled facility (12 h:12 h light-dark cycle) under standard food and water *ad libitum*. All experiments were approved by the National Institute on Alcohol Abuse and Alcoholism (NIAAA) Animal Care and Use Committee.

Induction of alcohol-associated HCC

Three different lines of *Aldh2*-deficient mice and their corresponding controls (10–12-week-old male mice) were injected intraperitoneally with CCl_4 (Sigma) (0.2 ml/kg in olive oil; 2× per week) for 28 weeks and fed 4% v/v ethanol-containing Lieber-DeCarli diet (Bio-Serv, Flemington, NJ) for the last 10 weeks out of 28 weeks.

Statistical analysis

Distributions of allele and genotype frequencies were analyzed using Chi-squared test. Logistic regression analyses to calculate an odds ratio (ORs) and 95% CIs were performed. For animal experiments, data are presented as the means ± SEM. Significance was evaluated by using Student's *t* test between 2 groups and one-way ANOVA for multiple comparisons. All p values <0.05 were considered to be statistically significant.

For further details regarding the materials used, please refer to the CTAT table and supplementary information.

Results

ALDH2 rs671 polymorphism is associated with an increased risk of HCC in patients with HBV and excessive alcohol consumption, but not in those without alcohol consumption

To study the association of *ALDH2* rs671 polymorphism with liver disease progression and cancer, we examined 646 patients with HBV and chronic liver disease, cirrhosis, and HCC with or without excessive alcohol consumption (Table S1). Because those who carried an A allele are associated with low ALDH2 activity, and the number of patients with homozygous AA genotype was small, we thus combined patients with GA and AA genotypes into one group GA/AA for ALDH2 deficiency in our analyses. Among these genotypes, there were no differences in

age, gender, antiviral drug treatment, and viral load (Table S1). As illustrated in Table 1, among patients with excessive alcohol consumption, the allele frequencies for GA/AA in patients with HBV-related HCC were statistically higher than those with HBV-related cirrhosis. In contrast, no distribution in the allele frequency for those with HBV-related cirrhosis and HBV-related HCC were observed among patients without alcohol consumption (Table 1 and Fig. S1A).

We next determined the effects of the *ALDH2* rs671 polymorphism on HCC staging among patients with HBV-related HCC stratified by excessive alcohol consumption. As illustrated in Table 1 and Fig. S1B, we observed the association between those with A allele (GA/AA) and advanced stages of HCC only in those with alcohol consumption. The allele frequencies for GA/AA in patients with HCC and excessive alcohol consumption who had Barcelona Clinic Liver Cancer (BCLC) stage C (54.83%) or D (32.26%) were significantly higher than those with BCLC stage B (3.23%, $p = 0.007$). We also used the logistic regression analysis to determine the association between those with A allele and HCC staging and found the ORs of 11.69 ($p = 0.01$) and 15.71 ($p = 0.003$), in patients with GA/AA genotypes presented with BCLC stage C and D, respectively, compared to those with stage B. Taken together, our data suggested that the risk of HCC development in patients with HBV and underlying cirrhosis only occurred in ALDH2-deficient patients who consumed alcohol. Additionally, ALDH2 deficiency is also associated with advanced stages of HCC among excessive alcohol drinkers.

Aldh2-deficient mice are more susceptible to CCl_4+EtOH-induced HCC

To model our clinical observation on HCC development in drinkers with underlying cirrhosis, we thus developed a mouse model of liver cancer by chronic CCl_4 administration for 18 weeks to induce severe liver fibrosis and followed by feeding 4% alcohol diet along with the injection of CCl_4 for an additional 10 weeks (denoted CCl_4+EtOH) (Fig. 1A). Hepatic histopathology from CCl_4+EtOH-treated mice showed fibrosis, steatosis, inflammation, hepatocyte ballooning degeneration, and tumor nodules (data not shown). Immunohistochemistry analyses detected the expression of HepPar-1, an immunohistochemical marker of HCC (Fig. S2A), suggesting CCl_4+EtOH treatment induces HCC, however, no tumor metastasis was found in other organs (Fig. S2B). These results suggest that the induction of CCl_4-mediated liver fibrosis followed by alcohol administration is a mouse model for alcohol-associated HCC.

Next, we tested this mouse model in 3 different lines of *Aldh2*-deficient mice, including *Aldh2*$^{-/-}$, *Aldh2*1/*2* knock-in, *Aldh2*$^{Hep-/-}$ mice, and their corresponding controls. *Aldh2*1/*2* mice were developed by replacing the mouse WT *Aldh2* allele with a mouse E487K mutant *Aldh2* allele, and resulted in marked loss of ALDH2 activity.[19] The body weight was decreased in *Aldh2*$^{-/-}$ and *Aldh2*1/*2* mice but not in *Aldh2*$^{Hep-/-}$ mice after alcohol feeding (Fig. S2C-D). Interestingly, serum alanine aminotransferase (ALT) levels were lower in all *Aldh2*-deficient mice after CCl_4+EtOH *vs.* WT mice (Fig. 1B). Collagen deposition and α-SMA expression were greater in

Table 1. Association of *ALDH2* polymorphisms and progression of HCC in patients.

Genotypes	HBV-related disease progression with excessive alcohol drinking			*P* values	Significant difference between groups
	[a]Chronic HBV without cirrhosis (n = 27)	[b]HBV-associated cirrhosis (n = 94)	[c]Patients with HBV-HCC (n = 116)		
GG	24 (12.50%)	83 (43.23%)	85 (44.27%)	0.012*	c *vs.* b
GA/AA	3 (6.67%)	11 (24.44%)	31 (68.89%)		
Genotypes	**HBV-related disease progression without excessive alcohol drinking**			***P* values**	**Significant difference between groups**
	[a]Chronic HBV without cirrhosis (n = 75)	[b]HBV-associated cirrhosis (n = 170)	[c]Patients with HBV-HCC (n = 164)		
GG	42 (17.72%)	107 (45.15%)	88 (37.13%)	0.213*	n.a.
GA/AA	33 (19.18%)	63 (36.63%)	76 (44.19%)		
Genotypes	**HCC staging with excessive alcohol drinking**				***P* values**	**Significant difference between groups**
	0 (n = 2)	**A (n = 18)**	**B (n = 23)**	**C (n = 49)**	**D (n = 24)**	
GG	2 (2.35%)	15 (17.65%)	22 (25.88%)	32 (37.65%)	14 (16.47%)	0.01*	C *vs.* B D *vs.* B
GA/AA	0 (0%)	3 (9.68%)	1 (3.23%)	17 (54.83%)	10 (32.26%)		
Genotypes	**HCC staging without excessive alcohol drinking**				***P* values**	**Significant difference between groups**
	0 (n = 5)	**A (n = 25)**	**B (n = 37)**	**C (n = 62)**	**D (n = 35)**		
GG	2 (2.27%)	16 (18.18%)	21 (23.86%)	34 (38.64%)	15 (17.05%)	0.51*	n.a.
GA/AA	3 (3.95%)	9 (11.84%)	16 (21.05%)	28 (36.84%)	20 (26.32%)		

*Chi-squared test was used for statistical evaluation.
ORs and 95% CIs for the GA/AA between c *vs.* b: 2.752 (95% CI 1.30–5.83, $p = 0.007$).
ORs and 95% CIs for the GA/AA genotype between stage C *vs.* B: 11.69 (95% CI 1.45–94.36, $p = 0.007$).
ORs and 95% CIs for the GA/AA genotype between stage D *vs.* B: 15.71 (95% CI 1.81–136.55, $p = 0.003$).
HCC, hepatocellular carcinoma; OR, odds ratio.

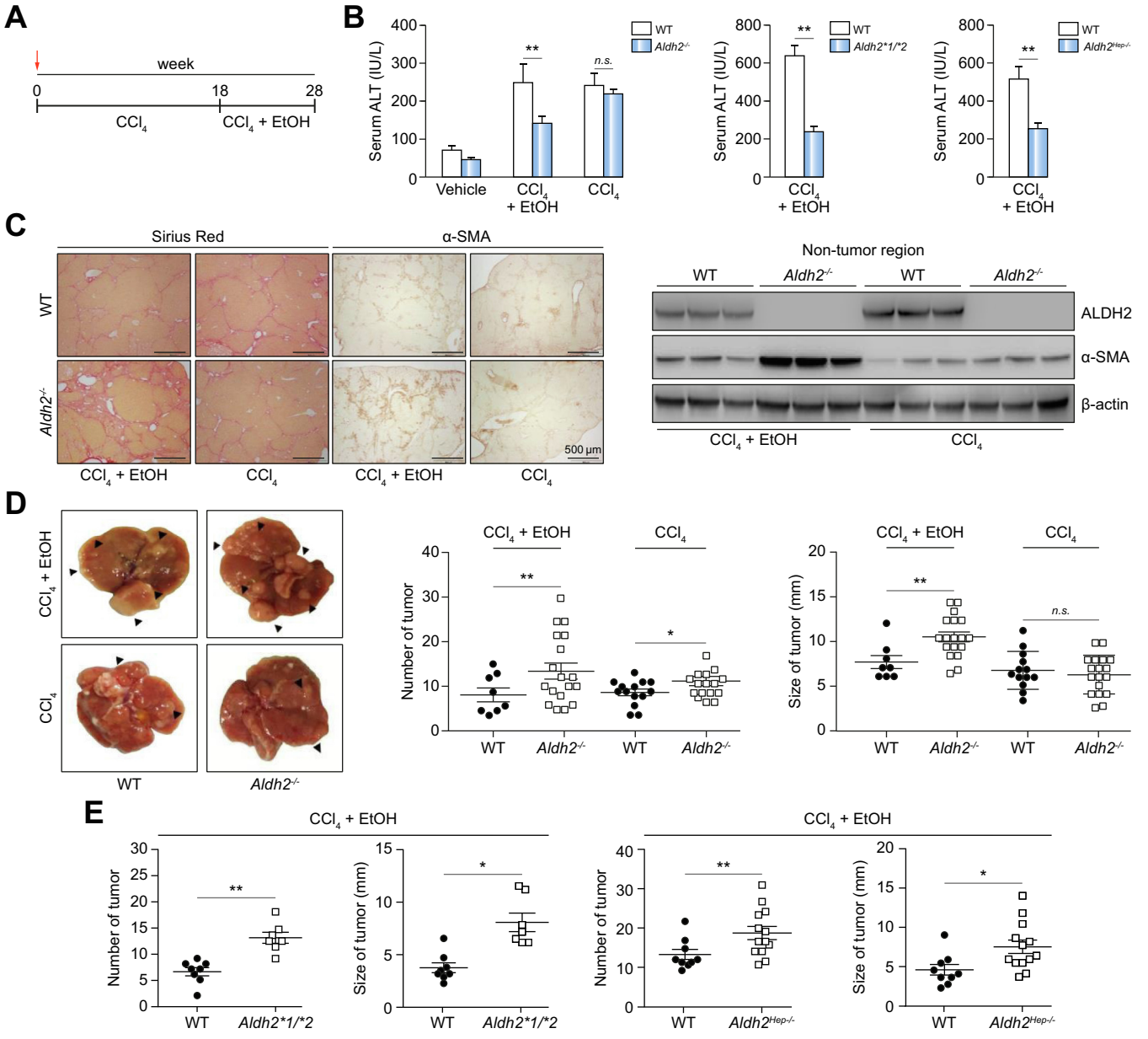

Fig. 1. *Aldh2* deficiency accelerates HCC development in a model of alcohol-associated HCC induced by chronic CCl_4+EtOH. (A) Three lines of *Aldh2*-deficient mice and their WT control mice were subjected to chronic CCl_4 administration for 28 weeks and ethanol feeding for the last 10 weeks. (B) Serum levels of ALT. (C) Liver tissues were stained with Sirius red and α-SMA antibody or tissues were subjected to western blotting analysis. (D) Representative gross findings of livers and HCC occurrence in $Aldh2^{-/-}$ mice. (E) The number and size (maximum diameter) of tumor after chronic CCl_4+EtOH. Student's *t* test was used for statistical evaluation (*p <0.05; **p <0.01). ALT, alanine aminotransferase; CCl_4, carbon tetrachloride; EtOH, ethanol; HCC, hepatocellular carcinoma; WT, wild-type. (This figure appears in colour on the web.)

CCl_4+EtOH-treated $Aldh2^{-/-}$ *vs.* WT mice, however, no differences were observed between these 2 groups after treatment with only CCl_4 (Fig. 1C). A greater degree of liver fibrosis was also observed in CCl_4+EtOH-treated *Aldh2*1/*2* and $Aldh2^{Hep-/-}$ mice compared to their controls (Fig. S3A-C). Furthermore, the number and size of tumors were greater in CCl_4+EtOH-treated $Aldh2^{-/-}$ mice *vs.* WT mice (Fig. 1D). In CCl_4 treated group, the number, but not the size of tumor was also higher in $Aldh2^{-/-}$ mice compared to WT mice (Fig. 1D). Moreover, CCl_4+EtOH-treated *Aldh2*1/*2* and $Aldh2^{Hep-/-}$ mice also had greater degree of liver fibrosis and number/size of tumors compared to their WT counterparts (Fig. 1E, Fig. S3A-C).

Liver progenitor cells (LPCs) with self-renewing abilities have been implicated in liver carcinogenesis through their transformation toward cancer stem cells and high ALDH activity is considered a functional marker of LPC.[21] Thus, we hypothesized whether *Aldh2* deficiency promotes LPC proliferation, which accelerates HCC progression. Because very few LPCs are detected in our CCl_4+EtOH model, we used 2 other models that significantly induce LPCs in the liver by feeding mice with choline-deficient ethionine supplemented (CDE) or 3,5-diethoxy carbonyl-1,4-dihydrocollidine (DDC) diet. Our data revealed that deletion of *Aldh2* did not affect LPC proliferation in CDE- or DDC-fed mice (Fig. S4A-C). Deletion of *Aldh1a1* neither

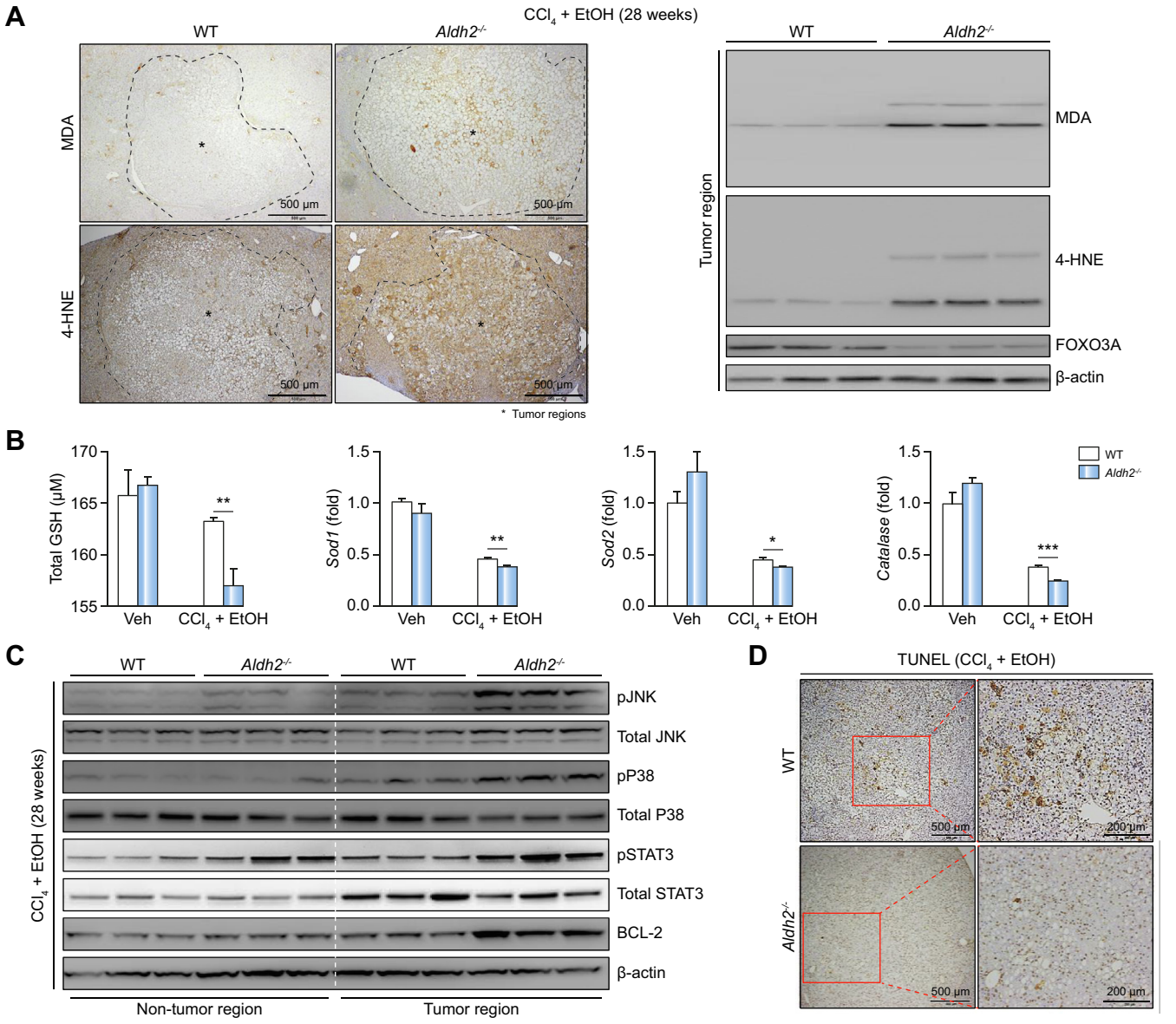

Fig. 2. Chronic CCl_4+EtOH activates oxidative stress and multiple oncogenic pathways in *Aldh2*-deficient mice. *Aldh2*$^{-/-}$ and WT mice were subjected to CCl_4+EtOH for 28 weeks as described in Fig. 1. (A) Liver tumor sections were subjected to immunohistochemistry and Western blotting analyses. (B) Liver tissues were subjected to Glutathione (GSH) assay and RT-qPCR analyses. (C) Liver non-tumor and tumor regions were subjected to Western blotting to evaluate indicated proteins involved in oxidative stress and cancer development. (D) Liver sections were subjected to TUNEL assay. Student's *t* test was used for statistical evaluation (**p* <0.05; ***p* <0.01; ****p* <0.001). CCl_4, carbon tetrachloride; EtOH, ethanol; HCC, hepatocellular carcinoma; RT-qPCR, quantitative reverse transcription PCR; WT, wild-type. (This figure appears in colour on the web.)

affected LPC proliferation in these models nor affected HCC progression induced by DEN+CCl_4 (Fig. S4A-C). Interestingly, the number of LPCs was lower in *Aldh2*$^{-/-}$ mice than in WT mice after alcohol feeding in both models (Fig. S4B). These data suggest that ALDH deficiency-associated HCC progression is unlikely mediated by stimulating LPCs.

Aldh2-deficient mice are more susceptible to CCl_4+EtOH-induced oxidative stress and activation of various oncogenic pathways

To understand the mechanisms underlying ALDH2 deficiency-associated HCC acceleration, we measured reactive oxygen species (ROS) in alcohol-associated hepatic tumor regions. As illustrated in Fig. 2A, immunohistochemistry analyses revealed that CCl_4+EtOH-treated *Aldh2*$^{-/-}$ mice expressed greater hepatic levels of lipid peroxidation markers including malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE) than those of WT mice, which was also confirmed by western blot analyses. In contrast, the expression of FOXO3A, an oxidative stress resistance protein, was lower in *Aldh2*$^{-/-}$ mice *vs.* WT mice. We further evaluated the expression of NRF2 and CTNNB1, which complexes with FOXO3A for anti-oxidant gene transcription.[22] Interestingly, expression of NRF2 protein and mRNA was not altered while expression of β-catenin (encoded by *CTNNB1*) was upregulated in CCl_4+EtOH-treated *Aldh2*$^{-/-}$ mice *vs.* WT mice (Fig. S5A). In addition, levels of hepatic glutathione (GSH), an important anti-oxidant, and the expression of ROS detoxification genes such including *Sod1*, *Sod2* and *Catalase 1* was lower in CCl_4+EtOH-treated *Aldh2*$^{-/-}$ mice *vs.* WT mice (Fig. 2B). Similar to these data, hepatic mRNA levels of *Sod1*, *Sod2* and

Catalase 1 were also downregulated in CCl_4+EtOH-treated *Aldh2*$^{Hep-/-}$ mice *vs.* WT mice (Fig. S5B). The mechanisms underlying downregulation of anti-oxidant genes in *Aldh2*-deficient mice are not clear, but it may be due to extensively produced oxidative stress in these mice as described above.

Oxidative stress is known to activate mitogen-activated protein kinase (MAPK) (*e.g.* JNK, and p38) and STAT3 phosphorylation, which is associated with HCC development.[10,11,13] Thus, we evaluated these kinase proteins in adjacent non-tumor and tumor regions in CCl_4+EtOH-treated HCC mouse model. As illustrated in Fig. 2C and Fig. S5C, pJNK, pP38, and pSTAT3 levels were significantly higher in *Aldh2*-deficient tumor tissues compared to those of WT. Furthermore, the number of TUNEL$^+$ apoptotic cells was lower, whereas *Bcl-2* mRNA levels were higher in CCl_4+EtOH- treated *Aldh2*$^{-/-}$ mice *vs.* WT mice (Fig. 2C-D, Fig. S5D).

TAZ and its target CTGF levels are higher in HCC from CCl_4+EtOH-treated *Aldh2*-deficient mice *vs.* WT mice

Hyperactivation of YAP/TAZ, 2 transcription factors mediating Hippo signaling, promotes cell proliferation while inhibiting cell death, ultimately leading to tumorigenesis.[14,15] To test whether YAP/TAZ is involved in HCC development, we measured the expression of YAP/TAZ and found that the levels of TAZ protein and mRNA were higher in HCC from CCl_4+EtOH-treated *Aldh2*$^{-/-}$ mice *vs.* WT mice; while YAP levels were comparable between these 2 groups (Fig. 3A-B). Greater TAZ protein levels in *Aldh2*$^{-/-}$ mouse HCC were further confirmed by immunohistochemistry analyses (Fig. 3C).

YAP and TAZ transcription factors regulate downstream gene expression when they are translocated from the cytoplasm to nuclei.[14,15] Here we found that nuclear TAZ protein levels but not nuclear YAP protein were much higher in CCl_4+EtOH-treated *Aldh2*$^{-/-}$ HCC compared to WT HCC (Fig. 3D), suggesting that TAZ but not YAP is activated in HCC from CCl_4+EtOH-treated *Aldh2*$^{-/-}$ mice. In agreement with TAZ activation, hepatic expression of the TAZ targeted gene *Ctgf* mRNA and protein levels was higher in tumor regions from CCl_4+EtOH *Aldh2*$^{-/-}$ mice *vs.* WT mice (Fig. 3E).

Aldh2 deficiency exacerbates DNA damage, oxidized mtDNA formation, hepatocyte senescence after CCl_4+EtOH treatment

To address the mechanism between *Aldh2* deficiency and alcohol-associated HCC, we further studied oxidative stress-induced DNA damage response by measuring the expression of γH2AX, a sensitive marker for damaged DNAs.[23] As illustrated in Fig. 4A, in non-tumor region, γH2AX protein levels were greater in *Aldh2*-deficient mice than those in WT mice; γH2AX protein levels in tumor regions were higher than in WT non-tumor region although no differences between

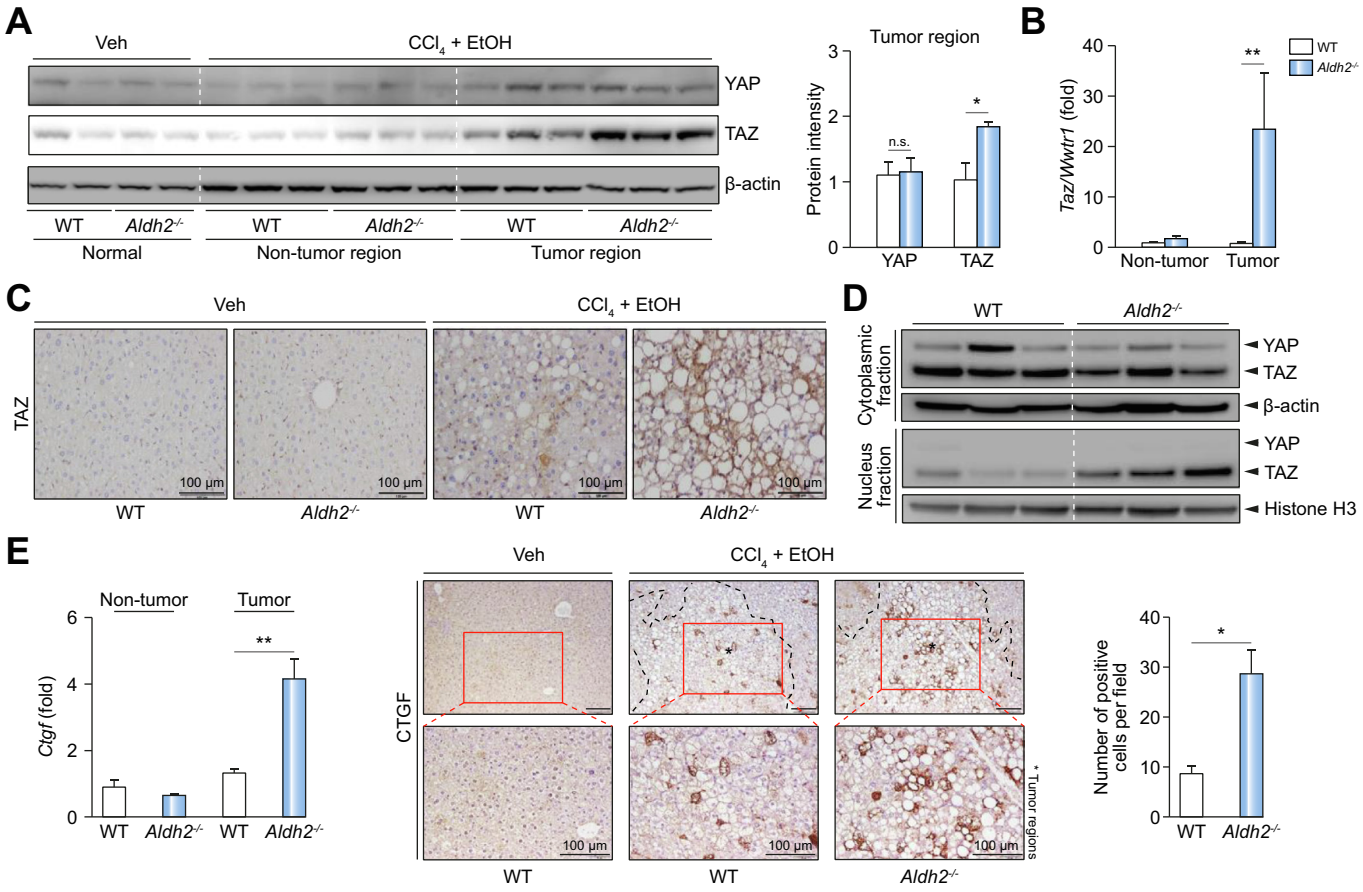

Fig. 3. The Hippo signal transducer TAZ and its target gene *Ctgf* expression are upregulated in CCl_4+EtOH-treated *Aldh2*$^{-/-}$ mice *vs.* WT mice. *Aldh2*$^{-/-}$ and WT mice were subjected to CCl_4+EtOH for 28 weeks. Liver tissues were collected for experiments. (A) Western blotting analysis. (B) RT-qPCR analysis. (C) Immunohistochemistry analysis. (D) Cytoplasmic/nucleus fractions were extracted and subjected to Western blotting. (E) Liver tissue sections were subjected to RT-qPCR analysis and CTGF immunohistochemistry. Student's *t* test was used for statistical evaluation (*p <0.05; **p <0.01). CCl_4, carbon tetrachloride; EtOH, ethanol; RT-qPCR, quantitative reverse transcription PCR; WT, wild-type. (This figure appears in colour on the web.)

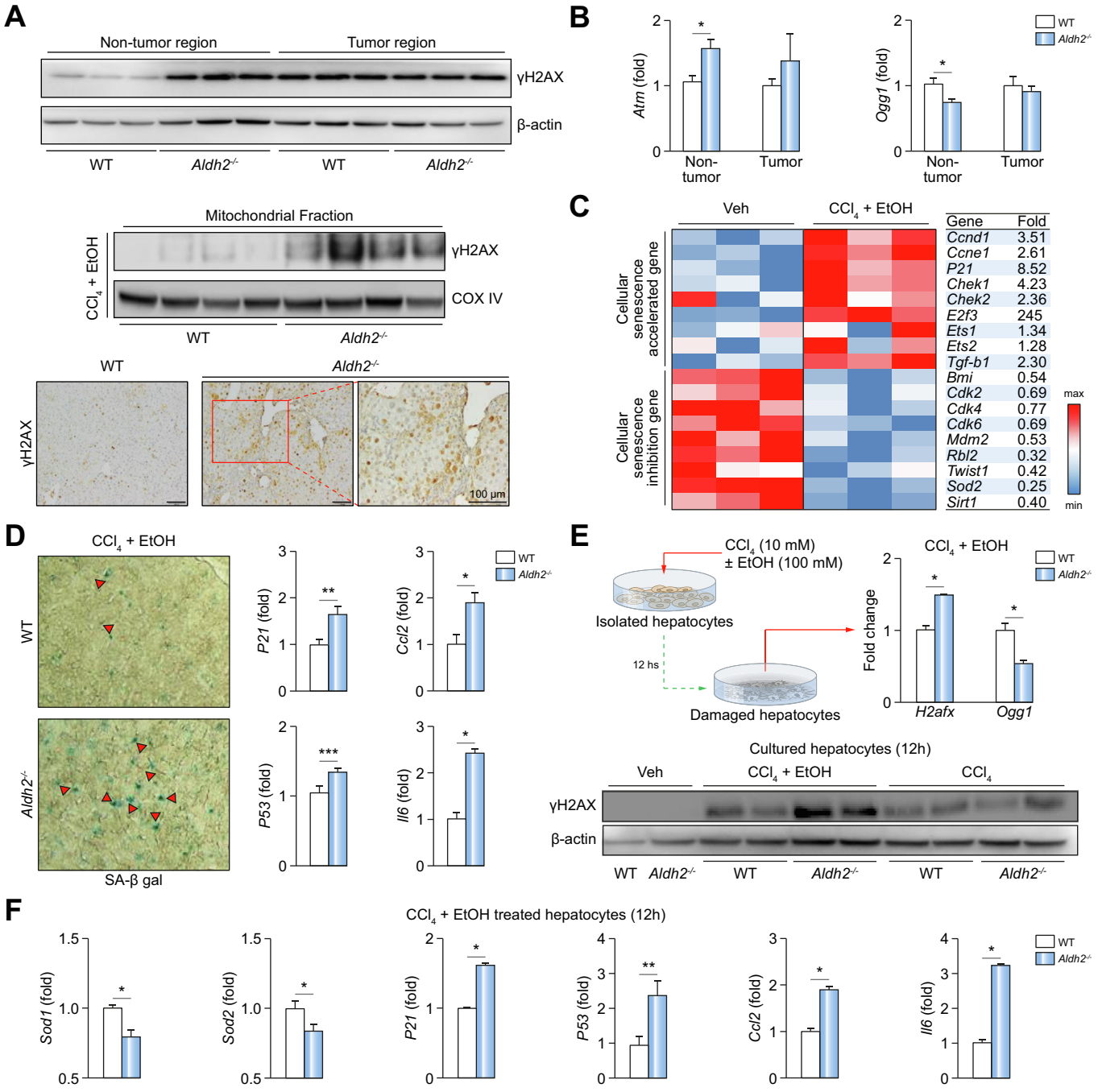

Fig. 4. DNA damage and cellular senescence are elevated in CCl_4+EtOH-treated *Aldh2*$^{-/-}$ mice *vs.* WT mice. *Aldh2*$^{-/-}$ and WT mice were subjected to CCl_4+EtOH treatment for 28 weeks. (A) Tissue lysates and liver tissue sections were subjected to Western blot and immunohistochemistry analysis of γH2AX. Mitochondrial fractions were also purified and subjected to Western blot analysis for γH2AX. (B) Liver tissues were subjected to RT-qPCR analysis. (C) Heat map of the mRNA expression changes of the cellular senescence pathways in the liver from vehicle-treated and chronic CCl_4+EtOH-treated mice. (D) Liver tissues were subjected to SA-β-gal staining (red arrowhead indicates positive staining) and RT-qPCR analyses. (E, F) Primary hepatocytes (WT and *Aldh2*$^{-/-}$) were treated with CCl_4 ± EtOH as indicated to induce hepatocyte damage and subjected to Western blotting and RT-qPCR analyses. Student's *t* test was used for statistical evaluation (**p* <0.05; ***p* <0.01; ****p* <0.001). CCl_4, carbon tetrachloride; EtOH, ethanol; RT-qPCR, quantitative reverse transcription PCR; WT, wild-type. (This figure appears in colour on the web.)

Aldh2$^{-/-}$ and WT groups. Immunohistochemistry analyses further confirmed greater γH2AX foci number in non-tumor regions from *Aldh2*$^{-/-}$ mice *vs.* WT mice. Moreover, the increased γH2AX expression in CCl_4+EtOH-treated *Aldh2*-deficient mice was detected in the mitochondrial fraction (Fig. 4A), but not in mice only treated with CCl_4 (Fig. S6A). Moreover, the expression of ataxia telangiectasia mutated (*Atm*), which encodes a protein that phosphorylates γH2AX in response to DNA double-strand breaks, was greater in *Aldh2*-deficient non-tumor regions; whereas the expression of oxoguanine DNA glycosylase 1 (*Ogg1*), excised base repairing gene, was downregulated (Fig. 4B).

Oncogenic/oxidative stress-derived DNA damage is a common mediator for cellular senescence[24] and is known to

promote the generation of neoplastic cells.[6] Thus, we examined cellular senescence and found that after CCl_4+EtOH, cellular senescence was significantly induced compared to normal liver tissues as illustrated by the alterations of several genes involved in cellular senescence pathways (Fig. 4C). In addition, the number of senescence-associated β-galactosidase (SA-β-gal) positive cells, and mRNA levels of *P21* and *P53* were higher in liver tissues from *Aldh2*$^{-/-}$ mice *vs.* WT mice after CCl_4+EtOH (Fig. 4D). Furthermore, mRNA expression of *Il1b*, *Il6* and *Ccl2*, known as common pro-inflammatory cytokines of senescence-associated secretory phenotypes (SASP),[25] were also higher in CCl_4+EtOH-treated *Aldh2*$^{-/-}$ mice *vs.* WT mice (Fig. 4D).

To model the effect of CCl_4+EtOH treatment *in vivo*, hepatocyte damage was induced *in vitro* by incubating with CCl_4+EtOH. Such treatment induced higher levels of γH2AX in *Aldh2*$^{-/-}$ hepatocytes than in WT hepatocytes; but no differences were observed in CCl_4 alone group (Fig. 4E). In addition, the expression of anti-oxidant mRNA levels (*Sod1, Sod2,* and *catalase 1*) was lower whereas expression of senescence-associated genes (*P21, P53, Ccl2,* and *Il6*) was higher in CCl_4+EtOH-treated *Aldh2*$^{-/-}$ hepatocytes than in WT hepatocytes (Fig. 4F, Fig. S6B).

Finally, CYP2E1 has been implicated in tumorigenesis by processing pro-carcinogen into carcinogen.[26] However, hepatic CYP2E1 protein levels were comparable between WT and *Aldh2*$^{-/-}$ mice (Fig. S6C), suggesting that the increased susceptibility of *Aldh2*-deficient mice to CCl_4+EtOH-induced HCC is not due to the CYP2E1 alternations.

Aldh2-deficient hepatocytes produce higher levels of oxidized mtDNA-enriched EVs than WT hepatocytes after CCl_4+EtOH

To further confirm that *Aldh2*-deficient hepatocytes are more susceptible to CCl_4+EtOH-induced DNA damage, we measured the major oxidative DNA-damage product 8-OhDG.[27] As illustrated in Fig. 5A, hepatic 8-OhDG levels in non-tumor tissues as well as serum 8-OhDG levels were significantly higher in CCl_4+EtOH-treated *Aldh2*$^{-/-}$ mice *vs.* WT mice.

EVs are known to play an important role in maintaining cellular homeostasis by excreting harmful mtDNA from cells,[28] and senescent cells are known to produce more EV.[29] Therefore, we hypothesized that *Aldh2*$^{-/-}$ hepatocytes remove these harmful oxidized mtDNA via EVs. First, we detected mtDNA in EVs and found that the EVs from CCl_4+EtOH-treated *Aldh2*$^{-/-}$ mice showed greater mtDNA copy numbers than those from WT mice (Fig. S7A). Second, the concentration of EVs from *Aldh2*$^{-/-}$ mouse serum or *Aldh2*$^{-/-}$ hepatocyte cultured medium was greater than that of WT (Fig. 5B, Fig. S7B). In addition, the size and morphology of isolated EVs were confirmed by NanoSight tracking and electron microscopy and western blot analysis of EV marker proteins (Hsp70 and TSG101) (Fig. 5B). Furthermore, the serum-derived-EVs from CCl_4+EtOH-treated mice were enriched with mtDNA (*Cyto C ox*, *D-loop*, and *Nd1*), but contained low levels of nuclear DNA (*Gapdh*) (Fig. 5C). Hepatocyte-specific protein CYP2E1 was also detected in EVs from CCl_4+EtOH-treated mice (Fig. S7B), suggesting that these EVs are mainly derived from damaged hepatocytes. Finally, flow cytometric analyses revealed that EVs from CCl_4+EtOH-treated *Aldh2*$^{-/-}$ mice or from *Aldh2*$^{-/-}$ hepatocytes contained much higher levels of 8-OhDG$^+$ EV fraction than those of WT mice or WT hepatocytes (Fig. 5D, Fig. S7C).

EV number was reported to be positively correlated with serum ALT levels.[30,31] However, our above data show that serum ALT levels were lower but EV concentration was higher in *Aldh2*$^{-/-}$ compared to WT mice after CCl_4+EtOH. The lower ALT levels in *Aldh2*$^{-/-}$ mice may be due to greater levels of BCL-2 expression in these mice *vs.* WT mice (as shown in Fig. 2C), the higher levels of EVs may be due to greater levels of ROS in *Aldh2*$^{-/-}$ mice *vs.* WT mice because anti-oxidant treatment reduced EV production in CCl_4+EtOH-treated hepatocytes (Fig. S7D).

The above data suggest that damaged hepatocytes can secrete damage DNA via EVs. Then we asked whether HCC can engulf these EVs. To validate the bio-distribution of EVs, we injected mice with fluorescent-labelled EVs and found that tumor regions had much higher fluorescent intensity than normal/non-tumor liver regions (Fig. 5E). Furthermore, EV engulfment related genes were significantly higher in *Aldh2*$^{-/-}$ mouse liver tumor tissues *vs.* non-tumor regions (Fig. 5F). In addition, after *in vitro* incubation with fluorescent-labelled EVs, HCC cells engulfed more EVs than primary hepatocytes, as demonstrated by greater reduction of the fluorescence-intensity in cultured medium (Fig. S7E). Collectively, these findings suggest that HCC cells engulf more EVs than primary hepatocytes.

Acetaldehyde and oxidized mtDNA-enriched EVs synergistically activate multiple oncogenic pathways in HCC

The above data suggest that *Aldh2*-deficient hepatocytes produce higher levels of oxidized mtDNA-enriched EVs, which can be engulfed by HCC cells. To examine the effects of these EVs on HCC, we treated HepG2 cells (with or without acetaldehyde) with serum EVs from CCl_4+EtOH-treated WT or *Aldh2*$^{-/-}$ mice. As illustrated in Fig. 6A, treatment with *Aldh2*$^{-/-}$ EVs and acetaldehyde synergistically induced higher levels of ROS production, H_2O_2 generation, and greater gene expression levels of *TAZ*, *CTGF*, and *BCL-2* compared to treatment with EVs from WT mice; whereas treatment with EVs or acetaldehyde alone had mild or no effects on these values.

Because oxidized mtDNA was enriched in these EVs, we next determined if oxidized DNA such as 8-OhDG DNA contributed to these effects by EVs observed in Fig. 6A. First, we extracted 8-OhDG$^+$ DNA from EVs and incubated with these DNA in HepG2 cells, and found 8-OhDG$^+$ DNA and acetaldehyde synergistically upregulated *TAZ* and *CTGF* mRNA levels in HepG2 cells (Fig. 6B). Second, we treated HepG2 with synthetic 8-OhDG DNA with or without acetaldehyde and found that synthetic 8-OhDG DNA treatment elevated the production of ROS and H_2O_2 levels in HepG2 cells in a dose-dependent manner in the presence of acetaldehyde (Fig. 6C). Such effects were not seen in primary hepatocytes (Fig. 6C) or murine hepatocyte cell line AML12 (Fig. S8). Finally, 8-OhDG and acetaldehyde synergistically upregulated the expression of *TAZ*, *CTGF* and *BCL-2* mRNAs (Fig. 6D) as well as the expression of TAZ and phosphorylated JNK, P38, and STAT3 proteins in HepG2 cells (Fig. 6E).

Discussion

In the current study, by analyzing our human data of *ALDH2* polymorphisms in 646 patients with HBV-related chronic liver disease, we found that ALDH2 deficiency conferred an increased risk of HCC development in cirrhotic patients with HBV who drank alcohol, but not in those who did not drink. To model this condition, we induced liver fibrosis in mice using CCl_4 followed by alcohol treatment and tested this model in 3 lines

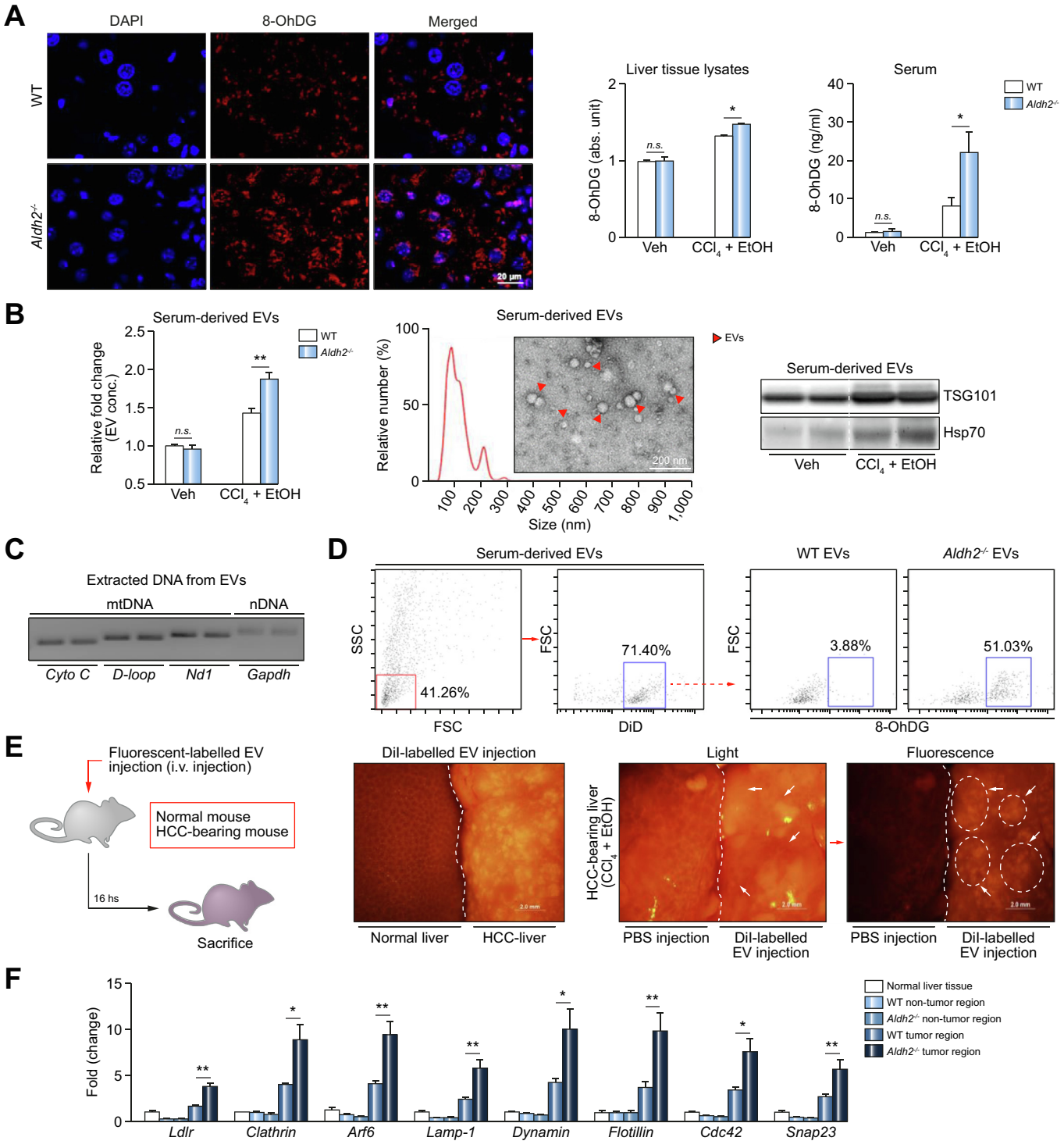

Fig. 5. *Aldh2*$^{-/-}$ mice produce a greater number of 8-OhDG enriched EVs, which can be engulfed by HCC cells, than WT mice after CCl_4+EtOH. *Aldh2*$^{-/-}$ and WT mice were subjected to CCl_4+EtOH for 28 weeks. (A) Liver tissues were subjected to immunofluorescence staining with 8-OhDG antibody. Hepatic and serum levels of 8-OhDG were assessed. (B) Concentration of serum-derived EVs was quantified using BCA assay and the characteristic of EVs was identified by Nanosight, transmission electron microscopy, and western blotting. (C) DNA fraction was extracted from serum-derived EVs and subjected to PCR amplification, and were loaded on 2% Agarose gel. (D) Isolated EVs were further subjected to flow cytometric analysis. (E) Schematic overview of the experimental design. Fluorescent-labelled EVs were introduced to C57BL/6N mice and CCl_4+EtOH-associated-HCC bearing mice. The mice were sacrificed after 16 hours, and livers were observed under fluorescent stereo microscope (White arrow heads or circles indicate tumor regions). (F) RT-qPCR analyses of EV engulfment related genes in the liver. Student's *t* test was used for statistical evaluation (**p* <0.05; ***p* <0.01). CCl4, carbon tetrachloride; EtOH, ethanol; EV, extracellular vesicle; HCC, hepatocellular carcinoma; RT-qPCR, quantitative reverse transcription PCR; WT, wild-type. (This figure appears in colour on the web.)

of *Aldh2*-deficient mice. Our data revealed that *Aldh2*-deficient mice were more susceptible to HCC development induced by CCl_4+EtOH. Such increased risk of HCC development in *Aldh2*-deficient mice after alcohol consumption is likely due to elevated acetaldehyde given acetaldehyde is a carcinogen that can cause DNA damage and mutagenesis, resulting in activation of the

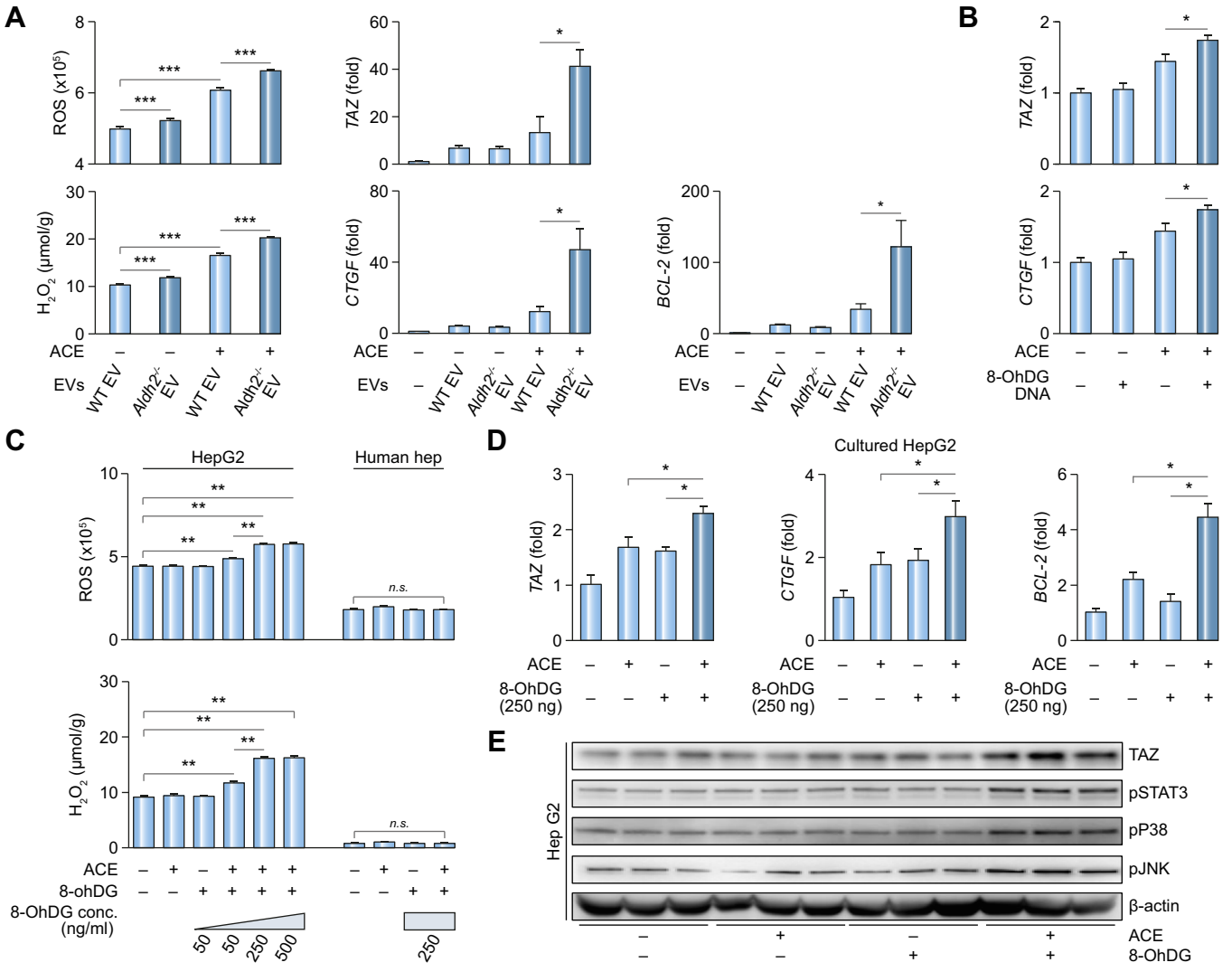

Fig. 6. Oxidized mtDNA-enriched EVs and acetaldehyde synergistically activate oxidative stress and multiple oncogenic pathways in HCC cells. (A, B) HepG2 cells were treated with acetaldehyde (100 μM) and serum-derived EVs (panel A) or purified 8-OhDG DNA from these EVs (panel B), and then subjected to OxiSelect intracellular ROS assay for ROS and H_2O_2 measurement, or RT-qPCR analysis. (C-E) HepG2 cells were treated with acetaldehyde (100 μM) and synthetic 8-OhDG (varying concentrations), and then subjected to OxiSelect intracellular ROS assay (panel C), RT-qPCR (panel D), and western blot analyses (panel E). One-way ANOVA was used for statistical evaluation (*p <0.05; **p <0.01; ***p <0.001). EV, extracellular vesicle; HCC, hepatocellular carcinoma; ROS, reactive oxygen species; RT-qPCR, quantitative reverse transcription PCR.

oncogenic pathway IL-6/STA3[18] and tumor transformation.[1] Both chronic CCl_4 administration and acetaldehyde have been shown to induce genomic DNA damage,[32,33] which may induce tumor initiation. Therefore, tumor initiation via genomic DNA damage induced by both CCl_4 and acetaldehyde likely contribute to the HCC development in this CCl_4+EtOH model in *Aldh2*$^{-/-}$ mice. In addition to these mechanisms, we also identified the novel mechanism by demonstrating the cross-talk between *Aldh2*-deficient hepatocytes and HCC via the transfer of oxidized mtDNA. *Aldh2*-deficient hepatocytes produce a greater number of EVs enriched with oxidized mtDNA than WT hepatocytes, which was likely due to CCl_4+EtOH induction of greater levels of DNA damage in these cells than in WT cells. Another mechanism may be because of greater levels of cellular senescence from *Aldh2*-deficient hepatocytes compared to WT hepatocytes given cellular senescence is known to accelerate EV secretion.[29]

Among the oxidized DNAs, 8-OhDG is one of the major products of DNA oxidation and has been widely used as a biomarker for oxidative stress and carcinogenesis.[27] Although 8-OhDG as a biomarker and risk factor for cancer development has been well documented, whether and how 8-OhDG contributes to alcohol-associated HCC development remain obscure. In the current study, we demonstrated that damaged *Aldh2*-deficient hepatocytes from CCl_4+EtOH exposure excreted much higher levels of 8-OhDG via EVs than WT hepatocytes. These harmful 8-OhDG-enriched EVs can be engulfed by HCC cells as demonstrated by *in vivo* and *in vitro* experiments with labelled EVs. The mechanisms by which HCCs engulfed more EVs than primary hepatocytes are not clear but may be due to the increased expression of EVs engulfment related genes in HCCs as demonstrated in this study. Importantly, we also demonstrated that treatment of HCC cells with 8-OhDG together with acetaldehyde induced activation of multiple oncogenic pathways that are known to promote HCC, including STAT3,[10,11] JNK,[12,13] TAZ,[14,15] and BCL-2.[16,17] Such activation is likely induced, at least in part, by oxidative stress induced by 8-OhDG and acetaldehyde given oxidative stress is

well-known to activate these oncogenic pathways.[34] Interestingly, 8-OhDG plus acetaldehyde-mediated activation of oxidative stress and multiple oncogenic pathways was only observed in HCCs but not in primary hepatocytes. The underlying mechanisms for this are not clear and it may be related to the fact that compared to HCC, primary hepatocytes express higher levels of anti-oxidant genes and ALDH2, which ameliorate ethanol-induced oxidative stress.

Another interesting finding from this study was that TAZ but not YAP was activated in *Aldh2*$^{-/-}$ tumors from CCl_4+EtOH-treated mice and in 8-OhDG+acetaldehyde-treated hepatocytes although both TAZ and YAP are downstream transcription factors in Hippo pathway with largely overlapping functions including promoting HCC progression.[14,15] A recent study revealed that oxidative stress-induced reversible S-glutathionylation at conserved cysteine residues within TAZ but not YAP, which subsequently increases TAZ protein stability and promotes TAZ activation.[35] This may explain why TAZ not YAP is markedly elevated in CCl_4+EtOH-treated *Aldh2*$^{-/-}$ HCC *vs.* WT HCC given greater ROS in *Aldh2*$^{-/-}$ than in WT HCC.

In summary, our study demonstrated that oxidized DNA-enriched EVs and acetaldehyde synergistically contribute to the development of alcohol-associated HCC, suggesting that inhibition of oxidized mtDNA-enriched EV production could be a novel therapeutic strategy for ameliorating alcohol-associated HCC in ALDH2-deficient individuals.

Financial support

The basic research work was supported by the intramural program of NIAAA, NIH (BG) and the clinical study of *ALDH2* polymorphisms and liver disease progression was supported by the Natural Science and Technology Major Project (2017ZX10202202 and 2018ZX10302206) and National Key Research Plan "Precision Medicine Research" key project (2017YFC0908103) (JN and YG). Dr. Wonhyo Seo was partially supported by the Korean Biomedical Scientist Fellowship Program (KBSFP) fellowship at the NIH.

Conflict of interest

The authors declare no conflicts of interest that pertain to this work.

Please refer to the accompanying ICMJE disclosure forms for further details.

Authors' contributions

YG, JS, HX, RU, JW, JN designed and conducted the study of *ALDH2* polymorphisms and HBV disease progression and HCC in patients. WS and YG designed and conducted the majority of mouse model and *in vitro* experiments. YH, DF, SP, AG, TR, SK, SH conducted some experiments. YC conducted analysis of extracellular vesicles. SL, YY, and JN analyzed the data and edited the paper. WS and BG wrote the paper. BG supervised the whole project.

Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jhep.2019.06.018.

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