ALDH2(E487K) mutation increases protein turnover and promotes murine hepatocarcinogenesis

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Mitochondrial aldehyde dehydrogenase 2 (ALDH2) in the liver removes toxic aldehydes including acetaldehyde, an intermediate of ethanol metabolism. Nearly 40% of East Asians inherit an inactive ALDH2*2 variant, which has a lysine-for-glutamate substitution at position 487 (E487K), and show a characteristic alcohol flush reaction after drinking and a higher risk for gastrointestinal cancers. Here we report the characterization of knockin mice in which the ALDH2(E487K) mutation is inserted into the endogenous murine *Aldh2* locus. These mutants recapitulate essentially all human phenotypes including impaired clearance of acetaldehyde, increased sensitivity to acute or chronic alcohol-induced toxicity, and reduced ALDH2 expression due to a dominant-negative effect of the mutation. When treated with a chemical carcinogen, these mutants exhibit increased DNA damage response in hepatocytes, pronounced liver injury, and accelerated development of hepatocellular carcinoma (HCC). Importantly, ALDH2 protein levels are also significantly lower in patient HCC than in peritumor or normal liver tissues. Our results reveal that ALDH2 functions as a tumor suppressor by maintaining genomic stability in the liver, and the common human ALDH2 variant would present a significant risk factor for hepatocarcinogenesis. Our study suggests that the *ALDH2*2* allele–alcohol interaction may be an even greater human public health hazard than previously appreciated.

ALDH2*2 polymorphism | Asian flush | alcohol metabolism | mouse model | liver cancer

Mitochondrial aldehyde dehydrogenase 2 (ALDH2) is essential for alcohol detoxification. It is the second enzyme in the major oxidative pathway of alcohol metabolism, removing acetaldehyde (ACE), a toxic intermediate product from ethanol metabolism (1). More than 500 million people worldwide, mostly in East Asia, have a G-to-A point mutation in their *ALDH2* gene (2, 3). This mutation results in a glutamic acid-to-lysine substitution at residue 487 (E487K) of the human ALDH2 protein (designated ALDH2*2). ALDH2*2 has significantly reduced ability to metabolize ACE (4, 5). Importantly, its activity is partially dominant-negative over that of the wild-type ALDH2*1, due to the structural alterations introduced by the mutation to the ALDH2 homotetramer complex (6). As a result, individuals with a heterozygous *ALDH2*2/2*1* genotype have less than half the wild-type activity, and *ALDH2*2/2*2* homozygotes have very low residual activity (7). Accumulated ACE can cause an alcohol flush reaction, commonly found in Asians with this variant after alcohol consumption (also called "Asian glow").

ACE binds to cellular proteins and DNA, leading to DNA damage and organ injury (8). Specifically, endogenous aldehydes are detrimental to hematopoietic stem cells that are defective in Fanconi anemia DNA repair (9, 10). As a result, Fanconi anemia patients with the *ALDH2*2* allele exhibit accelerated disease progression (11). ALDH2*2 can also increase the risk for gastrointestinal cancers, such as gastric carcinoma (12), esophageal cancer (13), and colon cancer (14). Despite the liver being the major organ of ethanol detoxification, the relationship between ALDH2*2 and the risk for liver cancer remains unclear (15, 16).

ALDH2 is highly conserved in humans and mice (17, 18), and several mouse models with modified ALDH2 activities have been developed (19). The closest model to the human ALDH2*2 polymorphism is the *Aldh2* knockout (KO) mouse, which expresses no protein or enzymatic activity (20). Although *Aldh2* KO mice are useful for investigating the impact of complete lack of ALDH2 activity (21), they fail to faithfully reproduce the structural and biochemical properties of ALDH2*2 in human physiology and pathology (21–23). In particular, ALDH2*2 is expressed with reduced but not loss of enzymatic activity and increased protein turnover (24).

To provide better mechanistic links between the ALDH2(E487K) mutation and human disease, we generated an ALDH2*2 knockin (KI) mouse. We observed impaired clearance of ACE from hepatocytes in these mutants after acute or chronic alcohol challenges. The ALDH2(E487K) mutation reduced total liver ALDH2 protein levels via a dominant-negative effect on protein stability, as has been documented for human tissues (24, 25). We also revealed a surprising role for ALDH2 as a liver tumor suppressor, raising the concern that this common human polymorphism may expose over 500 million carriers to greater risk of liver cancer.

Results

Generation of *Aldh2*^{E487K} KI Mice. The *Aldh2*^{E487K} KI mutation was generated through homologous recombination in mouse embryonic stem (ES) cells via introduction of a G-to-A substitution

Significance

About 40% of East Asians and over 500 million people worldwide carry a specific polymorphism, ALDH2*2, and exhibit "Asian flush" after alcohol drinking. We generated a mouse strain with this engineered polymorphism and demonstrated its resemblance to human carriers in terms of defective alcohol metabolism. With this model, we show that murine ALDH2*2 increases ALDH2 protein turnover and promotes chemical-induced liver tumor development. Importantly, ALDH2 is unstable in ALDH2*2 human liver samples and is significantly down-regulated in human liver tumors. Data from our mouse and clinical studies suggest that ALDH2 is a liver tumor suppressor and the ALDH2*2 polymorphism is a risk factor for liver cancer.

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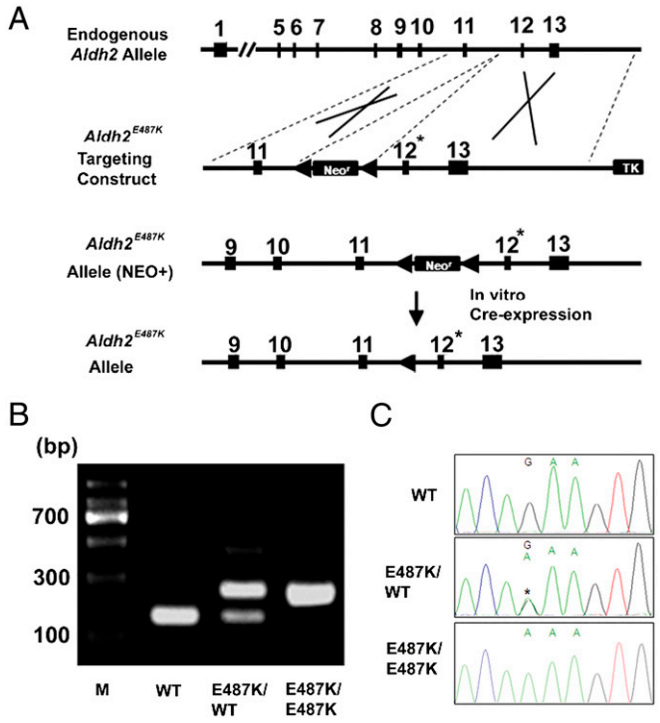

Fig. 1. Generation of *Aldh2*^{E487K} KI mice. (*A*) Schematic drawings of the murine *Aldh2* allele and targeting construct, *Aldh2*^{E487K} KI allele generated by homologous recombination in ES cells, and final *Aldh2*^{E487K} KI allele after Cre-mediated removal of the neomycin-resistance cassette. The dotted lines indicate regions of homology and asterisks indicate the altered exon. (*B*) PCR genotyping of WT, *Aldh2*$^{E487K/+}$, and *Aldh2*$^{E487K/E487K}$ mice ("M" indicates molecular weight marker). (*C*) Confirmation of the E487K mutation by direct sequencing of PCR-amplified genomic DNA; *indicates the position of the nucleotide alteration producing the E487K mutation.

in exon 12 of the endogenous Aldh2 locus, producing a missense mutation orthologous to the human ALDH2(E487K) mutation (Fig. 1*A*). The KI allele was confirmed by PCR detection of the remaining *loxP* site (Fig. 1*B*) after Cre-mediated deletion of the neomycin-resistance cassette and by direct sequencing of either genomic DNA (Fig. 1*C*) or cDNA amplified from transcribed ALDH2 mRNA. *Aldh2*^{E487K} KI mice generated from these ES cells were born at the expected Mendelian ratio, had a normal life span, and were healthy and fertile.

Reduced Enzymatic Activity in *Aldh2*^{E487K} KI Hepatocytes. ALDH2 activity was assessed in primary hepatocytes isolated from *Aldh2*^{E487K} KI mice (Fig. 2*A*). Hepatocytes from mice carrying one *Aldh2*^{E487K} allele retained only 44% of ALDH2 activity compared with wild-type mice. In contrast, *Aldh2*$^{E487K/E487K}$ homozygous hepatocytes produced hardly detectable ALDH2 activity. Because ALDH2 catalyzes oxidation of ACE to acetic acid in alcohol metabolism, the cellular kinetics of ACE clearance was quantitated by liquid chromatography mass spectrometry in cultured hepatocytes with isotopically labeled [^{13}C]ACE (Fig. 2 *B* and *C*). Primary hepatocytes of heterozygous *Aldh2*^{E487K} mice cleared [^{13}C]ACE more slowly than those of wild-type mice, whereas homozygous *Aldh2*^{E487K} hepatocytes failed to clear [^{13}C]ACE altogether. These results indicate that the ALDH2(E487K) mutant is a loss-of-function enzyme defective in metabolizing its substrate in the liver.

Increased Ethanol Intoxication in *Aldh2*^{E487K} KI Mice. To investigate the effects of the *Aldh2*^{E487K} mutation on alcohol metabolism, we acutely challenged wild-type and KI mice with ethanol (2, 4, or 8 g/kg) and monitored serum ACE levels, behavioral abnormality, and survival. When exposed to low-dose ethanol (2 g/kg), heterozygous and homozygous *Aldh2*^{E487K} mice exhibited similarly slow ACE clearance but no behavioral abnormality compared with wild-type mice (Fig. 3 *A* and *D*). After 4 g/kg ethanol, homozygous *Aldh2*^{E487K} mice failed to clear serum ACE (Fig. 3*B*), and both groups of *Aldh2*^{E487K} mice showed genotype-dependent mild to severe ataxia (Fig. 3*E*). High-dose ethanol (8 g/kg) abolished ACE clearance and caused severe behavioral abnormalities including loss of righting reflex in both heterozygous and homozygous *Aldh2*^{E487K} mice (Fig. 3 *C* and *F*). High-dose ethanol (8 g/kg) was lethal in all three groups, with the homozygous mice most sensitive (Fig. S1), consistent with their impaired ACE clearance.

To study the chronic effects of ethanol consumption, we treated wild-type and *Aldh2*^{E487K} mice with 4 g/kg ethanol (every day) for 6 wk. This dose was chosen to induce remarkable differences in serum ACE clearance and behavioral abnormality without acute lethal effect. Some lethality was observed in both heterozygous and homozygous *Aldh2*^{E487K} mice, beginning at 2 wk (Fig. 4*A*). Moreover, all KI mice lost more body weight than wild-type controls during ethanol treatment (Fig. 4*B*). Whole-blood smears revealed significantly reduced numbers of white cells in the KI mice relative to wild-type controls (Fig. 4*C* and Fig. S2*A*), indicating possible injury of the hematopoietic system caused by chronic ethanol treatment. Furthermore, we observed hepatocyte apoptosis by histology analysis and cleaved caspase 3 staining on liver sections of *Aldh2*^{E487K} mice (Fig. 5*D*). However, no inflammatory or necrotic changes were identified in other organs of *Aldh2*^{E487K} mice, including esophagus and stomach (Fig. S2*B*). Taken together, these data suggest that mice with even one copy of the ALDH2 variant are more susceptible to ethanol intoxication and organ injury.

Increased ALDH2 Protein Turnover in *Aldh2*^{E487K} KI Mice. It has been observed in prior studies that ALDH2 protein levels are very low in ALDH2*2 liver (24, 26). The ALDH2(E487K) variant increases overall ALDH2 protein turnover through a dominant but unknown mechanism (24). We examined the levels of ALDH2 protein in lysates of 20 human liver surgical specimens (Fig. S3). Consistent with previous reports, humans with single-allele *ALDH2*^{E487K} mutations expressed only about 27% of liver ALDH2 protein relative to wild-type individuals (Fig. 5 *A* and *B*). Likewise, one allele of *Aldh2*^{E487K} in mice caused an 85% decrease in liver ALDH2 protein, whereas two mutant alleles led to a nearly complete loss of ALDH2 protein (Fig. 5 *D* and *E*). *ALDH2* mRNA levels were not different in humans or mice (Fig. 5 *C* and *F*), suggesting a posttranscriptional regulation. Cycloheximide chasing experiments on purified mouse hepatocytes indicate that total ALDH2 protein is less stable in heterozygous or homozygous KI cells compared with wild type (Fig. 5 *G* and *H*), revealing a reduced stability of the ALDH2(E487K) protein itself and a dominant effect on wild-type ALDH2 protein.

Reduced ALDH2 Levels in Human HCC Samples. Given the defects in clearing ACE and detoxifying ethanol by *ALDH2*^{E487K} liver, we reasoned that ALDH2*2 humans might be more prone to develop liver cancer. We analyzed the genetic polymorphism of *ALDH2*^{E487K} mutations in HCC samples and observed a 40% mutation rate (17 of 43) comprising 5% homozygous and

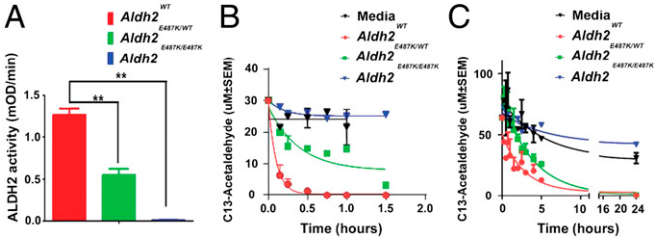

Fig. 2. Significantly reduced ALDH2 enzymatic activity in *Aldh2*^{E487K} KI mouse hepatocytes. Primary hepatocytes from WT and *Aldh2*^{E487K} KI mice were analyzed for ALDH2 activity in vitro (mean ± SEM; $^{**}P < 0.01$; $n = 6$) (*A*) and for ACE clearance over a short (*B*) or long (*C*) time course (mean ± SEM; $n = 3$).

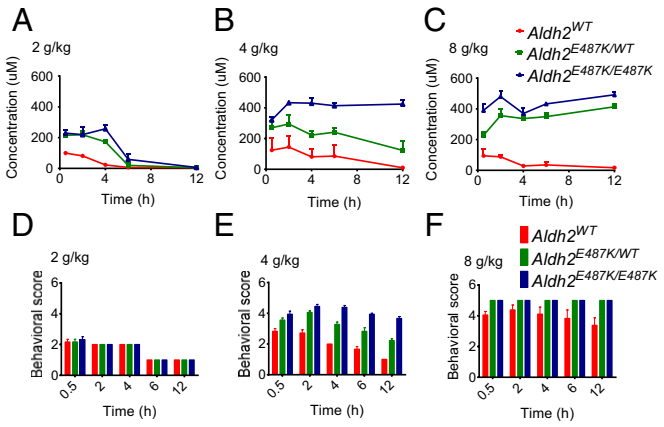

Fig. 3. Defective alcohol metabolism in *Aldh2*^{E487K} KI mice after acute ethanol challenge. Serum ACE concentrations from WT and *Aldh2*^{E487K} KI mice acutely challenged with ethanol at 2 g/kg (*A*), 4 g/kg (*B*), or 8 g/kg (*C*) over 12 h (mean ± SEM; $n = 3$). Behavioral scores of mice subjected to 2 g/kg (*D*), 4 g/kg (*E*), or 8 g/kg (*F*) ethanol over the same time course (mean ± SEM; $n = 6$).

35% heterozygous mutations (Fig. S4). The mutation rate of *ALDH2*^{E487K} in HCC samples was close to the 40% mutation rate found in normal livers (Fig. S3), suggesting that within our limited and random tumor samples no obvious correlation exists between *ALDH2*^{E487K} mutation and HCC incidence.

However, surprisingly, ALDH2 protein levels were significantly reduced in almost all HCC samples we examined compared with matching peritumor liver tissues by Western blot analysis (Fig. 6). This decrease was found in six out of nine wild-type and six out of seven ALDH2*2 mutant patients compared with their matching peritumor tissues (Fig. 6). In addition, immunostaining revealed that ALDH2 expression was also reduced in all mouse hepatomas from either a chemical-induced model (27) or a spontaneous genetic model (28) compared with immediately neighboring nontumor liver tissues (Fig. S5). These data suggest that ALDH2 might play a tumor suppressor role in the liver, independent of the ALDH2 polymorphism.

Increased Hepatocarcinogenesis in *Aldh2*^{E487K} KI Mice. Because *ALDH2*^{E487K} KI mice express significantly reduced ALDH2 protein in the liver (Fig. 5), we investigated their susceptibility to liver tumor development. We induced liver tumors in wild-type and *Aldh2*^{E487K} KI mice by injecting diethylnitrosamine (DEN) at postnatal day (P) 14 and examined the livers after 8 mo (Fig. 7*A*). Mice of all three groups developed liver tumors (Fig. 7*B*); however, *Aldh2*^{E487K} mutant mice developed a greater number and a larger volume of liver tumors than wild-type controls (Fig. 7 *C* and *D*). Serum alanine transaminase (ALT) activity was also higher in *Aldh2*$^{E487K/WT}$ mice and further elevated in *Aldh2*$^{E487K/E487K}$ mice (Fig. 7*E*), indicating more severe liver injury. Finally, liver histology revealed more focal hepatic lesions in *Aldh2*^{E487K} KI mutants (Fig. 7*F*, *Upper*), and immunostaining for phosphorylated histone H2AX demonstrated a substantially higher degree of DNA damage in the mutants (Fig. 7*F*, *Lower*). We conclude that *ALDH2*^{E487K} KI mice are more susceptible to carcinogen-induced liver tumor development.

Discussion

***Aldh2*^{E487K} KI Mice Model Human Deficiency in Alcohol Metabolism.** ALDH2 catalyzes ACE metabolism and plays important roles in the pathogenesis of cardiovascular diseases, diabetes, neurodegenerative diseases, and cancer (19). The ALDH2*2 polymorphism is the most common ALDH2 deficiency in humans (29, 30), and several transgenic mouse models have been developed to investigate the roles of this enzyme (31). These models include *Aldh2* knockout (21), ALDH2 overexpression (32) and *Aldh2*^{E487K} overexpression (33) mice. Here we generated a mouse strain with a mutation in murine ALDH2 corresponding to the E487K substitution in human ALDH2. The same mouse strain was independently generated while this manuscript was under preparation, and was shown to accumulate ACE in the blood upon ethanol challenge and exhibit heightened pain response (34). Here, we provide a comprehensive description of ACE metabolism in these heterozygote and homozygote mutant mice.

First, in addition to increased blood ACE levels, *Aldh2*^{E487K} KI mice were more susceptible to ethanol toxicity than wild-type mice after acute alcohol challenge, due to the impaired clearance of ACE by their hepatocytes. Second, upon chronic alcohol exposure, the KI mice lost more weight than controls, and homozygotes died early. The number of white blood cells in KI mice was reduced dramatically, indicating possible injury of the hematopoietic system caused by ACE accumulation. Despite the increased apoptotic cells in the liver of the KI mice, no significant injury was observed in the esophagus and stomach, likely because the 6-wk treatment was too short to induce injury. Interestingly, we also observed that all KI mice exhibited hyperpigmentation on the feet (Fig. S6). This cutaneous change is also found in chronic alcoholics, who often demonstrate hyperpigmentation and erythema, particularly on the legs (35, 36).

Dominant-Negative Effect of ALDH2(E487K) on ALDH2 Protein Stability. It is thought that ALDH2 deficiency in human ALDH2*1/*2 heterozygotes results from a dominant-negative effect of the mutant enzyme on the catalytic activity of the holoenzyme containing the wild-type enzyme (25). However, we observed a significant reduction of ALDH2 protein levels and shortened half-lives of all ALDH2 proteins in the livers of both human and mouse heterozygotes. The protein is barely detectable in mouse homozygotes. No difference of ALDH2 RNA levels was found between mutants and wild types, suggesting a posttranscriptional mechanism regulating the turnover of the mutant ALDH2 protein as well as the wild-type protein.

To investigate the mechanism underlying the instability of ALDH2(E487K) protein, we tested whether the lysine residue introduced in the mutant ALDH2 might make it more susceptible to polyubiquitination and therefore proteasome-mediated degradation.

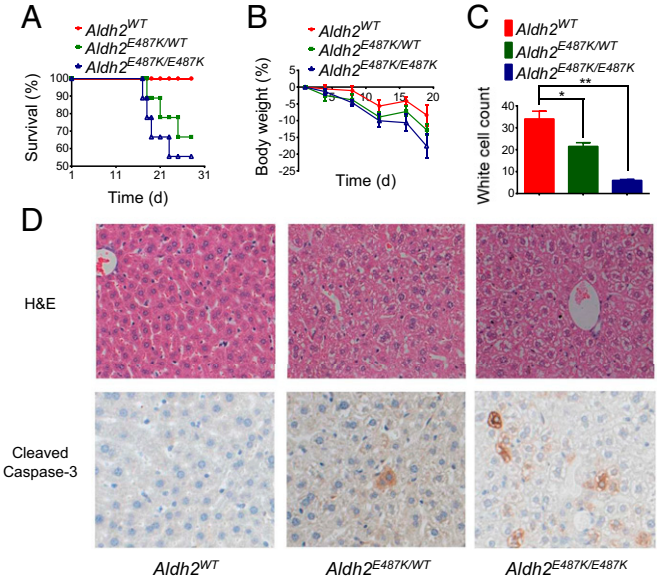

Fig. 4. Increased sensitivity of *Aldh2*^{E487K} KI mice to damage induced by chronic ethanol challenge. (*A* and *B*) Survival (*A*) and body weight (*B*) of WT and *Aldh2*^{E487K} KI mice treated with 4 g/kg ethanol for 6 wk (mean ± SEM; $n = 9$). (*C*) White blood cell counts from whole-blood smears of chronically treated mice (mean ± SEM; $*P < 0.05$, $**P < 0.01$; $n = 3$). (*D*) Histology and cleaved caspase 3 staining of treated mouse liver sections (400×).

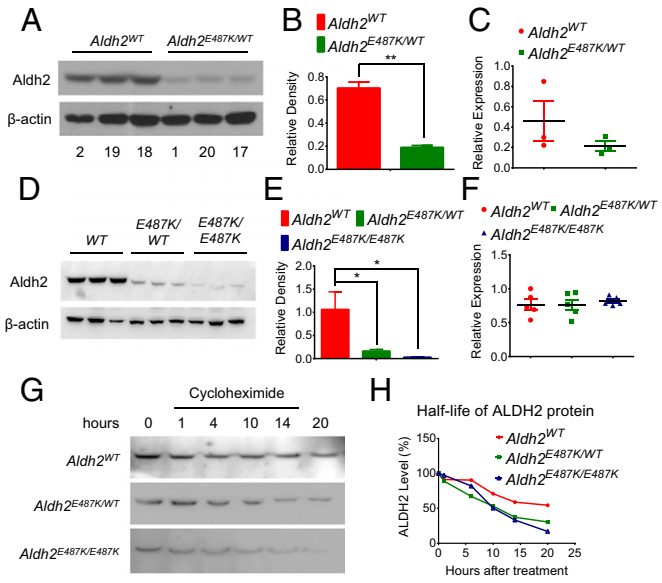

Fig. 5. Reduced levels and stability of ALDH2 protein in liver of ALDH2*2 humans and *Aldh2*^{E487K} KI mice. (*A* and *B*) Representative Western blot (*A*) and quantification (*B*) relative to β-actin of human liver ALDH2 with the indicated genotypes. (*C*) Measurement of *ALDH2* mRNA by real-time quantitative PCR in these human samples. (*D* and *E*) Representative Western blot (*D*) and quantification (*E*) normalized to β-actin of ALDH2 in heterozygous and homozygous *Aldh2*^{E487K} KI mouse liver. (*F*) Measurement of *ALDH2* mRNA by real-time quantitative PCR in these mouse samples. (*G* and *H*) Western blot (*G*) and quantification (*H*) of ALDH2 in primary hepatocytes from *Aldh2*^{E487K} KI mice after cycloheximide treatment to determine the half-life of ALDH2 proteins (mean ± SEM; $*P < 0.01$; $**P < 0.001$; $n = 3$).

ALDH2(E487K) mutant protein did not exhibit increased ubiquitination in vitro, and treating primary hepatocytes from mutant mice with a proteasome inhibitor did not result in increased ALDH2 protein levels (Fig. S7). Although the precise mechanism awaits further investigation, new routes of pharmaceutical interference might be directed toward increasing ALDH2 protein stability in addition to its enzymatic activity (19, 37).

Increased Hepatocarcinogenesis by ALDH2(E487K). People with the ALDH2*2 polymorphism are reported to be at a greater risk of developing esophageal and other digestive tract cancers (12–14, 38), attributable to ACE-induced DNA damage and genome instability (39, 40). *Aldh2*^{E487K} KI mice did not exhibit obvious damage in the esophagus or stomach after 2 mo of daily ethanol challenge, judged by histology evaluation. No tumors or dysplastic nodules were found in any organs of these mice, and deletion of one copy of the *Trp53* gene, encoding the tumor suppressor p53, in the KI mice did not accelerate the tumorigenesis. Longer chronic exposure to ethanol may be required to evaluate ethanol- and ACE-mediated tumorigenesis in this model.

The relationship between the ALDH2 polymorphism and liver cancer risk remains unclear (15, 16), despite the fact that liver is the major organ for alcohol metabolism and ACE production and that chronic ethanol challenge causes liver damage (Fig. 5*B*). In this study, we analyzed the *ALDH2*^{E487K} mutation in human samples and found that the mutation rate in HCC samples (39%) was similar to that in normal livers (40%) and within the range reported for East Asians, suggesting no obvious direct relationship between the *ALDH2*^{E487K} mutation and HCC incidence. However, we observed lower ALDH2 protein levels in HCC samples compared with matching peritumor liver tissues independent of the ALDH2 polymorphism. To test ALDH2 as a potential HCC tumor suppressor, we induced HCC in *Aldh2*^{E487K} mutant and wild-type mice with DEN, a chemical carcinogen causing hepatocyte DNA damage. Compared with controls, *Aldh2*^{E487K} mutant mice developed a greater number and larger volume of HCCs, and the effect was more pronounced in homozygote mutants than in heterozygote mutants. The DNA damage response as indicated by γH2AX staining and liver injury measured by ALT elevation was also greatly increased in the mutants bearing larger HCC loads. How this polymorphism cross-talks with the known HCC-promoting signaling network awaits future investigation (41).

Our data suggest that the ALDH2*2 population might be more susceptible to HCC if exposed to liver-damaging agents, as is observed in the *Aldh2*^{E487K} mutant mice exposed to DEN. In support, the ALDH2 polymorphism was reported to be a significant risk factor for HCC development and recurrence in Japanese hepatitis C virus patients (42, 43). The ALDH2 polymorphism may also be a risk factor in other subpopulations of HCC patients when their disease etiology is closely analyzed in association with their *ALDH2* genotype. Another explanation for the human and mouse difference in HCC incidence is that human carriers of the ALDH2*2 polymorphism might refrain from drinking alcohol due to "alcohol flush," and hence are less likely to be subjected to alcohol-induced liver damage and cancer than noncarriers. This population might be more prone to develop liver cancer due to their ALDH2 deficiency if their alcohol consumption were equivalent to that of noncarriers. Therefore, grouping all HCC patients in an association analysis might miss the differential contribution of this mutation to the disease.

Based on our clinical and mouse data, we conclude that ALDH2 may protect liver cells from DNA damage induced by chemical agents such as aldehydes and DEN. Although the ALDH2*2 polymorphism itself does not directly lead to liver cancer, it reduces ALDH2 protein levels and causes enzyme deficiency in the liver, leading to inefficient detoxification of aldehydes and accumulation of cancer-causing mutations.

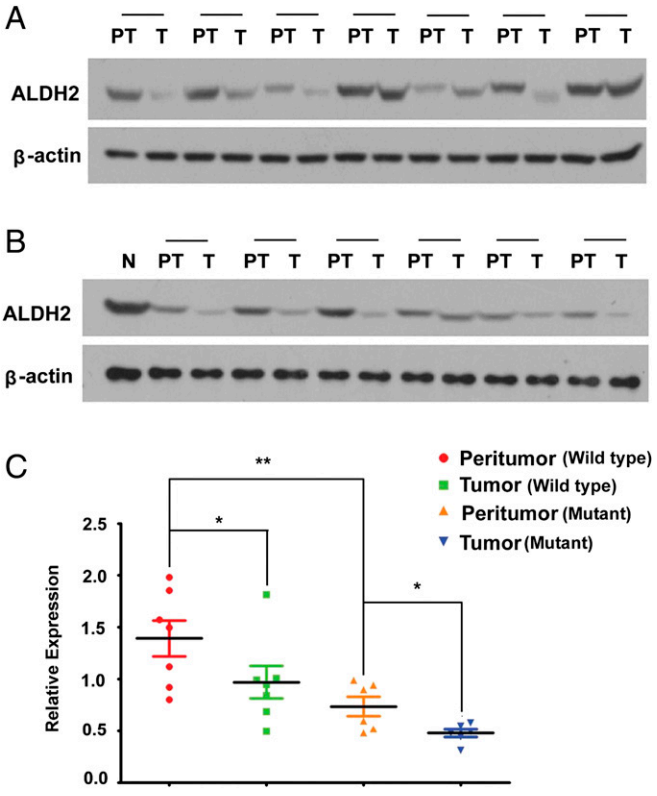

Fig. 6. Reduced ALDH2 expression in human liver tumor samples independent of the ALDH2 polymorphism. (*A* and *B*) Western blot of ALDH2 in liver tumor (T) and peritumor (PT) tissues from (*A*) ALDH2*1/2*1 patients or (*B*) ALDH2*2/2*1 patients. N, normal WT liver. (*C*) Quantification of ALDH2 levels normalized to β-actin (mean ± SEM; $*P < 0.05$, $**P < 0.01$).

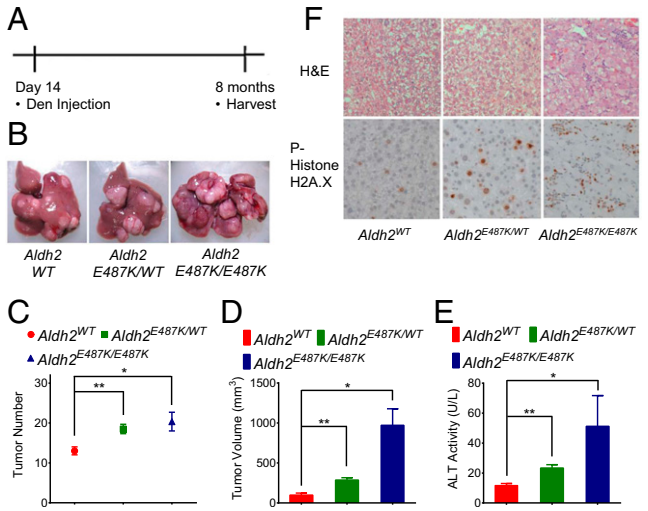

Fig. 7. Accelerated liver tumor development in DEN-induced *Aldh2*^{E487K} KI mice. (*A*) Outline of the experimental protocol. (*B*) Representative photographs of livers dissected from WT or KI mice 8 mo after DEN treatment. (*C* and *D*) Total number (*C*) and maximal volumes (*D*) of liver tumors (>0.5 mm) from WT and KI mice. (*E*) Serum ALT activity to estimate liver injury. (*F*) Histology and phospho-H2AX staining (400×) of liver tissue sections (mean ± SEM; $*P < 0.05$, $**P < 0.01$; $n = 4$).

Methods

Generation of *Aldh2*^{E487K} Knockin Mice. The *Aldh2*^{E487K} knockin targeting vector was assembled using genomic fragments of the mouse *Aldh2* gene including a 2.0-kb 5′ arm and a 6.4-kb 3′ arm retrieved from 129S6/SvEvBAC DNA (origin) via an *Escherichia coli*-based chromosome engineering system (44), a *loxP*-flanked neomycin-resistance cassette (Neo^r), and a thymidine kinase gene (TK) as negative selection for genomic integration. The 3′ arm contains murine *Aldh2* exon 12 bearing the E487K mutation, which was created by PCR-based mutagenesis. The linearized targeting construct was electroporated into 129S6/SvEv mouse embryonic stem cells. Following homologous recombination in ES cells, a Cre recombinase expression vector was transiently transfected to delete the Neo^r cassette. Targeted ES cells were identified by PCR and sequencing and injected into mouse blastocysts to produce chimeras. The chimeric mice were bred to C57BL/6J mice to generate heterozygous and homozygous *Aldh2*^{E487K} mice. Mice were genotyped using PCR primers spanning the position of the residual *loxP* site (5′-CGGGAATTGAACTTGGTAGCCAG-3′ and 5′-GCGTAAGGCATGCGCCATCAC-3′), generating a 169-bp PCR product in WT mice and a 263-bp PCR product in KI mice. Initial litters were analyzed for the presence of *Aldh2*^{E487K} by PCR and sequencing using the primers 5′-CCTGAGCCGAATGCTTTTAAG-3′ and 5′-CTCACGCTCCTTACTGGAC-3′. Wild-type littermates of *Aldh2*^{E487K} mice served as controls for all analyses.

Mice were bred and maintained in a barrier facility under pathogen-free conditions at WuXi AppTec. Mice were housed on a 12-h/12-h light/dark cycle and given food and water ad libitum. Mice were older than 8 wk at the beginning of the experiments and were used in accordance with protocols approved by the Institutional Animal Care and Use Committee.

Patient Samples. The use of human samples was approved by the local ethics committee (Institutional Review Board of Sir Run Run Shaw Hospital) and included informed consent. HCC tissues were collected from patients undergoing liver resection for HCC, and normal liver tissues were collected from patients undergoing laparoscopic surgery for intrahepatic calculi at the Sir Run Run Shaw Hospital (Zhejiang University). All tissues were stored at −80 °C.

Genetic Polymorphism of ALDH2 in Human Tissues. Genomic DNA was extracted by a MiniBEST Universal Genomic DNA Extraction Kit (TaKaRa). The human *ALDH2* gene was analyzed by PCR using primers spanning exon 12 (5′-TCAAATTACAGGGTCAACTGCTA-3′ and 5′-GCCCCCAACAGACCCCAATC-3′). The 222-bp fragment was digested with Eco57I (Fermentas) and subjected to agarose gel electrophoresis. The wild-type *ALDH2* allele yielded two fragments of 132 and 90 bp, and the *ALDH2*^{E487K} allele yielded a single product of 222 bp (45).

Primary Mouse Hepatocytes. Hepatocytes were isolated from mice by the modified collagenase method as previously described (46). Mice were anesthetized using 1% pentobarbital sodium, and livers were perfused with buffer solution (10 mM Hepes, 3 mM KCl, 130 mM NaCl, 1 mM NaH_2PO_4, 10 mM glucose) containing 0.5 mM EGTA (pH 7.4) and then with buffer solution containing 5 mM $CaCl_2$ and 100 U/mL collagenase type IV (Sigma) at a rate of 2 mL/min. Livers were isolated, and cells were dissociated and washed with PBS. Hepatocytes were plated at 1×10^5 viable cells per cm^2 on rat tail collagen-coated (BD) tissue-culture dishes in DMEM, 15% FBS (vol/vol), 100 U/mL penicillin, and 100 μg/mL streptomycin and kept in a humidified cell-culture incubator at 37 °C and 5% CO_2 until use the following day.

Reverse-Transcription Reaction and Quantitative Real-Time PCR. Total RNA was extracted with TRIzol reagent (Invitrogen). RNA was reverse-transcribed into cDNA with a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). cDNAs were quantified using real-time PCR in a StepOne Plus system according to the standard SYBR Green PCR Kit protocol (Applied Biosystems) in triplicate. β-*actin* was used to normalize the amount of total mRNA. Relative expression was calculated with the comparative CT method. Primer sequences were as follows: *Aldh2*: 5′-gctgggctgacaagtaccat-3′ and 5′-ttgatcaagttggccacgta-3′; β-*actin*: 5′-GACGGCCAGGTCATCACTAT-3′ and 5′-CTTCTGCATCCTGTCAGCAA-3′; *ALDH2*: 5′-TCAAATTACAGGGTCAACTGCTA-3′ and 5′-GCCCCCAACAGACCCCAATC-3′; β-*ACTIN*: 5′-CTTAGTTGCGTTACACCCTTTC-3′ and 5′-CACCTTCACCGTTCCAGTTT-3′.

Western Blotting. Tissues or cell samples were homogenized in RIPA buffer (Sigma) containing protease inhibitor mixture (Roche), incubated on ice for 30 min, and centrifuged for 15 min at 12,000 × *g*. Equal amounts of proteins were separated by SDS/PAGE, transferred to nitrocellulose membranes (Invitrogen), and incubated overnight at 4 °C with primary antibodies. Immunoblots were visualized with horseradish peroxidase (HRP)-conjugated secondary antibody (Santa Cruz Biotechnology; 1:2,000) using enhanced chemiluminescence (Pierce Biotechnology). Primary antibodies were ALDH2 (goat polyclonal; Santa Cruz Biotechnology; 1:500), p21 (mouse monoclonal; Cell Signaling Technology; 1:1,000), and β-actin (rabbit monoclonal; Cell Signaling Technology; 1:1,000).

Cycloheximide Treatment. Cycloheximide (20 μg/mL; Sigma) was added to primary mouse hepatocytes to block protein synthesis. Total cell lysates were subjected to Western blotting using an anti-ALDH2 antibody. The relative band intensity was quantified using Quantity software (1D component of ImageQuant LAS 4010; GE Healthcare).

ALDH2 Activity Assay. The activity of ALDH2 was analyzed using the colorimetric Mitochondrial Aldehyde Dehydrogenase (ALDH2) Activity Assay Kit (Abcam; ab115348) according to the manufacturer's protocol.

ACE Metabolism in Primary Hepatocytes. Freshly thawed primary mouse hepatocytes (1×10^6) were plated in Corning T25 flasks and incubated overnight at 37 °C and 5% CO_2 in 1:1 DMEM:F12 media with 15% (vol/vol) FBS. Universally labeled [^{13}C]ACE was added to each flask to a concentration of 75 μM, and plug caps were used to prevent ACE loss. A flask containing media and ACE alone (no cells) was used as a control for ACE evaporation. At 0, 0.25, 0.5, 0.75, 1, 1.5, 2, 2.5, 3, 4, 5, and 24 h, duplicate 60-μL aliquots of media were frozen on dry ice and stored at −80 °C before liquid chromatography mass spectrometry (LCMS) quantitation.

LCMS Measurement of ACE. ACE concentrations were determined through derivatization with dinitrophenylhydrazine (DNPH) using butyraldehyde-DNPH (Supelco) as an internal standard. Tissue-culture media or blood (60 μL) was deproteinized by addition of an equal volume of cold acetonitrile (ACN) containing 10 μg/mL butyraldehyde-DNPH and centrifuged for 30 min at 3,500 × *g* at 4 °C. One hundred microliters of supernatant was derivatized by the addition of 30 μL of 0.75 μg/mL freshly prepared DNPH in ACN and 10 μL of 1 M citric acid (pH 4.0). After 30 min at 25 °C, the reaction was quenched with 120 μL of 0.1% formic acid in water. Samples were analyzed by direct injection onto a Waters ACQUITY UPLC system and Applied Biosystems API 4000 Q MS system. Chromatographic separation was performed using a Kinetex C18 30 × 2.1 mm 100A column at a flow rate of 500 μL/min, using water + 0.1% formic acid as buffer A and ACN + 0.1% formic acid as buffer B. The gradient was 0–0.5 min 95% buffer A, to 3.5 min 5% buffer A, to 4 min at 5% buffer A, return to 95% buffer A at 4.5 min, and reequilibration of the column to 5 min at 95% buffer A. [^{13}C]ACE-DNPH and butyraldehyde-DNPH were monitored using the following multiple reaction monitoring transitions, respectively: [224.960/46, declustering potential (DP) −40, entrance potential (EP) −10, collision energy (CE) −42, collision cell exit potential (CXP) −5] and (250.958/46.0, DP −55, EP −10, CE −52, CXP −5). Quantitation was performed using a [^{13}C]ACE standard curve prepared in parallel, and rate constants of ACE consumption were determined by fitting time/concentration values to a first-order decay model.

Ethanol Challenge. For acute exposure, mice were administered ethanol (Sigma) in water by gavage once at doses of 2, 4, and 8 g/kg. For chronic exposure, mice were treated with 4 g/kg ethanol daily for 6 wk.

Behavioral Score. After acute ethanol challenge, mouse behaviors were observed and scored according to the following system: 1, rearing; 2, sedation; 3, mild ataxia (dysfunction of hind limbs); 4, severe ataxia (inability to move, body shaking); 5, loss of righting reflex.

Blood Smear. EDTA-anticoagulated blood samples were collected and thick/thin smears were prepared. Smears were air-dried, thin smears were fixed in methanol, and thick/thin smears were stained by Giemsa (pH 7.2) (Sigma). Slides were rinsed with water, air-dried, and examined under a microscope. White blood cells were counted in five random high-power (200×) fields and are given as the number of cells per field.

Histology and Immunohistochemistry. Mouse tissues were fixed overnight at 4 °C in PBS-buffered 4% (wt/vol) paraformaldehyde and embedded in paraffin. Five-micrometer cross-sections were prepared and stained with hematoxylin and eosin. For immunohistochemistry, tissue sections were blocked with blocking buffer and incubated with antibodies against anti-phosphohistone H2AX (Cell Signaling Technology; 1:50).

Tumor Induction and Analysis. P14 mice were injected intraperitoneally with 25 mg/kg DEN (Sigma) (27) and euthanized after 8 mo. Mouse livers were isolated and separated into individual lobes. Externally visible tumors (>0.5 mm) were counted and volumes were measured by stereomicroscopy.

ALT Activity. Blood samples anticoagulated by EDTA-K_2 were collected and centrifuged at 3,000 × *g* for 10 min at 4 °C. The activity of serum ALT was analyzed using a Reitman–Frankel ALT Activity Assay Kit (Jiancheng).

Statistical Analysis. All results are presented as the means ± SEM of at least three independent experiments; each was performed in duplicate or triplicate. Data were analyzed using the two-sided Student's *t* test and one-way analysis of variance (ANOVA) with appropriate multiple comparison correction as required, using Prism 5.0 software (GraphPad). Differences were considered significant at $P < 0.05$ ($^{*}P < 0.05$, $^{**}P < 0.01$).

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