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ORIGINAL ARTICLE

Protective effects of Alda-1, an ALDH2 activator, on alcohol-derived DNA damage in the esophagus of human ALDH2*2 (Glu504Lys) knock-in mice

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Abstract

Alcohol consumption is the key risk factor for the development of esophageal squamous cell carcinoma (ESCC), and acetaldehyde, a metabolite of alcohol, is an alcohol-derived major carcinogen that causes DNA damage. Aldehyde dehydrogenase2 (ALDH2) is an enzyme that detoxifies acetaldehyde, and its activity is reduced by _ALDH2_ gene polymorphism. Reduction in ALDH2 activity increases blood, salivary and breath acetaldehyde levels after alcohol intake, and it is deeply associated with the development of ESCC. Heavy alcohol consumption in individuals with _ALDH2_ gene polymorphism significantly elevates the risk of ESCC; however, effective prevention has not been established yet. In this study, we investigated the protective effects of Alda-1, a small molecule ALDH2 activator, on alcohol-mediated esophageal DNA damage. Here, we generated novel genetically engineered knock-in mice that express the human _ALDH2*1_ (wild-type allele) or _ALDH2*2_ gene (mutant allele). Those mice were crossed, and human _ALDH2*1/*1_, _ALDH2*1/*2_ and _ALDH2*2/*2_ knock-in mice were established. They were given 10% ethanol for 7 days in the presence or absence of Alda-1, and we measured the levels of esophageal DNA damage, represented by DNA adduct (N^2-ethylidene-2′-deoxyguanosine). Alda-1 significantly increased hepatic ALDH2 activity both in human _ALDH2*1/*2_ and/or _ALDH2*2/*2_ knock-in mice and reduced esophageal DNA damage levels after alcohol drinking. Conversely, cyanamide, an ALDH2-inhibitor, significantly exacerbated esophageal DNA adduct level in C57BL/6N mice induced by alcohol drinking. These results indicate the protective effects of ALDH2 activation by Alda-1 on esophageal DNA damage levels in individuals with _ALDH2_ gene polymorphism, providing a new insight into acetaldehyde-mediated esophageal carcinogenesis and prevention.

Introduction

Esophageal squamous cell carcinoma (ESCC) is the disease with high morbidity and mortality (1,2). Alcohol consumption is the major risk factor for ESCC, and acetaldehyde, a metabolite of ethanol, is considered to play a central role in alcohol-related esophageal carcinogenesis (3–5). Acetaldehyde causes various DNA damages, such as DNA adducts including N^2-ethylidene-2′-deoxyguanosine (N^2-ethylidene-dG) (6) and N^2-ethyl-2′-deoxyguanosine (N^2-Et-dG) (7), single- and/or double-strand

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Abbreviation

ALDH2	aldehyde dehydrogenase 2
cDNA	complementary DNA
ESCC	esophageal squamous cell carcinoma
N^2-Et-dG	N^2-ethyl-2′-deoxyguanosine
UTR	untranslated region

breaks, point mutations, sister chromatid exchanges and DNA–DNA cross-links (8–13). 'Acetaldehyde associated with alcohol consumption' is designated as a definite carcinogen for the esophagus (14).

Acetaldehyde is detoxified to acetic acid by aldehyde dehydrogenase 2 (ALDH2) mainly in the liver (15). *ALDH2* gene has two major alleles, *ALDH2*1* (active ALDH2) and *ALDH2*2* (inactive ALDH2) (16). *ALDH2*2* (rs671) allele, also known as Glu504Lys, encodes the ALDH2 protein, which is defective at metabolizing acetaldehyde (17). Combination of *ALDH2*1 and ALDH2*2 alleles* makes up three genotypes with various enzymatic activity; wild-type (*ALDH2*1/*1*), heterozygous (*ALDH2*1/*2*) and homozygous (*ALDH2*2/*2*) (18,19). As *ALDH2*2* acts in a dominant negative manner (20), hepatic activity of heterozygous (*ALDH2*1/*2*) and homozygous (*ALDH2*2/*2*) mutant *ALDH2* is less than 10% and 0%, respectively, compared with that of wild-type (*ALDH2*1/1*) *ALDH2* (21–23). *ALDH2*2/*2* carriers do not tolerate alcohol and few of them habitually drink alcoholic beverages, meanwhile, *ALDH2*1/*2* carriers may drink various amount of alcohol (24,25). Previous epidemiological analysis showed that *ALDH2*1/*2* carriers with heavy alcohol consumption are at high risk for ESCC (26–28). According to a meta-analysis, *ALDH2*1/*2* carriers have a 7.12-fold increased risk of ESCC, compared with *ALDH2*1/*1* carriers (29). Moreover, alcoholics with the *ALDH2*1/*2* carriers have a 13.5-fold increased risk of ESCC, compared with *ALDH2*1/*1* carriers (30). Thus, the risk of ESCC in *ALDH2*1/*2* carriers rises according to the amount of alcohol consumption (31). Of note, about 70% of ESCC patients in East Asian countries such as Japan or Taiwan are revealed to have *ALDH2*2* allele carriers (32,33).

Alcohol intake increases acetaldehyde concentrations in the blood, saliva and the breath, and *ALDH2* gene polymorphisms affect those levels (34–36). Importantly, salivary acetaldehyde concentration reaches a high level in individuals with *ALDH2*1/*2* after alcohol consumption (37). Consequently, high acetaldehyde-containing saliva can be directly exposed to the pharynx and esophagus and may induce various acetaldehyde-mediated DNA damages in these individuals. Indeed, esophageal DNA damage levels in *ALDH2* knockout mice were much higher than those in control *ALDH2* wild-type mice after drinking 10% ethanol for 2 months (38,39).

Thus, impaired ALDH2 activity due to *ALDH2* gene polymorphism is considered to be deeply involved in esophageal carcinogenesis. Therefore, restoration of ALDH2 activity might be beneficial to *ALDH2*1/*2* carriers in ESCC prevention. At present, a small molecule Alda-1 has been identified as an ALDH2 activator (40,41). Here, we hypothesized that Alda-1 may reduce esophageal DNA injury in the *ALDH2*2* allele carriers with alcohol consumption. To verify that hypothesis, we generated novel genetically engineered knock-in mice that express the human *ALDH2*1* and/or *ALDH2*2* and investigated the effects of Alda-1 on these knock-in mice.

The aim of this study was to clarify whether Alda-1 has protective effects against alcohol-derived DNA damage in the esophagus of *ALDH2*2* allele carriers.

Materials and methods

Mouse preparation

Age-matched control C57BL/6N male mice, which carry the wild-type *Aldh2* gene, were purchased from Charles River Laboratories Japan (Yokohama, Japan). All experiments conformed to the relevant regulatory standards and were approved by the institutional animal care and use committee of Kyoto University (Med Kyo 16196). To obtain tissues from mice, they were euthanized painlessly under anesthesia with diethyl ether inhalation followed by cervical dislocation. For the measurement of DNA adduct levels, esophageal tissues were frozen in liquid nitrogen and stored at −80°C until use. Liver tissues were collected in liquid nitrogen and stored at −80°C for analysis of ALDH activity.

Generation of genetically engineered knock-in mice that express the human *ALDH2*1* (wild-type allele) or *ALDH2*2* gene (mutant allele)

Human *ALDH2*1* or *ALDH2*2* knock-in mice in C57BL/6N background were generated by homologous recombination in TransGenic (Fukuoka, Japan) in accordance with the institutional guidelines. We designed targeting vector to replace mouse *Aldh2* gene with human *ALDH2*1* or *ALDH2*2* complementary DNA (cDNA) (Figure 1A). The design of the vector construction is summarized in Figure 1B. As 5′ homologous arms, we used a 2.6k base pair (bp) mouse genomic fragment containing 5′ untranslated region (UTR) of exon 1 of *Aldh2* gene. As 3′ homologus arms, we used a 5.1k bp fragment containing exon 3–7 of *Aldh2* gene. These genomic fragments were amplified by PCR from RENKA ES cell genomic DNA (42).

Mouse *Aldh2* 5′ UTR sequence was amplified by PCR from RENKA ES cell genomic DNA and cloned into a vector, named pUC118 generated by TransGenic, followed by human *ALDH2* (*ALDH2*1* or *ALDH2*2*) cDNA fragment (1554 bp) and bovine growth hormone polyA signal (5′ UTR + cDNA + polyA).

3′ homologous arm was amplified by PCR and cloned into a vector, named pPND2.1 generated by TransGenic, which contains lox-P flanked PGK_neo cassette (phosphoglycerate kinase 1 promoter-driven neomycin resistant gene) as a positive selection marker and MC1_DTA cassette (polyoma enhancer/herpes simplex virus thymidine kinase promoter driven diphtheria toxin A gene) as a negative selection marker.

Then, PCR-amplified 5′ homologous arm and 5′ UTR + cDNA + polyA were subcloned into the plasmid with 3′ homologous arm, PGK_neo cassette and MC1 DTA cassette. Resulting targeting vector contains MC1_DTA cassette, 5′ homologous arm, human *ALDH2* (*ALDH2*1* or *ALDH2*2*) cDNA fragment, polyA, lox-P flanked PGK_neo cassette and 5.1 kb 3' homologous arm. Thus, *ALDH2*1* or *ALDH2*2* targeting vector was generated. To amplify the fragment from genomic DNA, primer sets as shown in Table 1 were used.

This targeting vector was linearized and introduced into RENKA ES cells (C57BL/6N) by electroporation. After selection using neomycin, the resistant clones were isolated, and their DNAs were screened for homologous recombination by PCR using following primer set: *sc_5AF2*: 5′-ACC ATC CAT TCA AGG TAA AGT TCC -3′ and *neo_108r*: 5′-CCT CAG AAG AAC TCG TCA AGA AG-3′. PCR positive ES clones were expanded, and isolated DNAs were further analyzed by PCR amplification using following primer sets: *sc_5AF2* and *neo_108r* for 5′ amplification, *sc_3AR3*: 5′-CAG GCA CAG GTT ACT ACT CTT CC-3′ and *neo_MS*: 5′-ATT CGC AGC GCA TCG CCT TCT ATC GCC TTC -3′ for 3′ amplification. Homologous recombination of these clones was also confirmed by genomic Southern hybridization probed with neomycin-resistant gene.

Homologous recombinant ES cell clones were aggregated with ICR 8 cell embryos to generate chimeric mice. Germline transmitted heterozygous *ALDH2* knock-in mice (F1 heterozygous *ALDH2*1* or *ALDH2*2* knock-in mice) were obtained by crossing chimeric mice with a high contribution of the RENKA background with C57BL/6N mice. Targeted allele was identified by PCR with the following primer sets: *sc_5AF2* and *neo_108r.*

F1 heterozygous *ALDH2*1* or *ALDH2*2* knock-in mice were crossed each other to obtain F2 homozygous *ALDH2*1* or *ALDH2*2* knock-in mice. The F2 homozygous mice were crossed with B6.Cg-Tg (CAG-Cre) CZ-MO2Osb

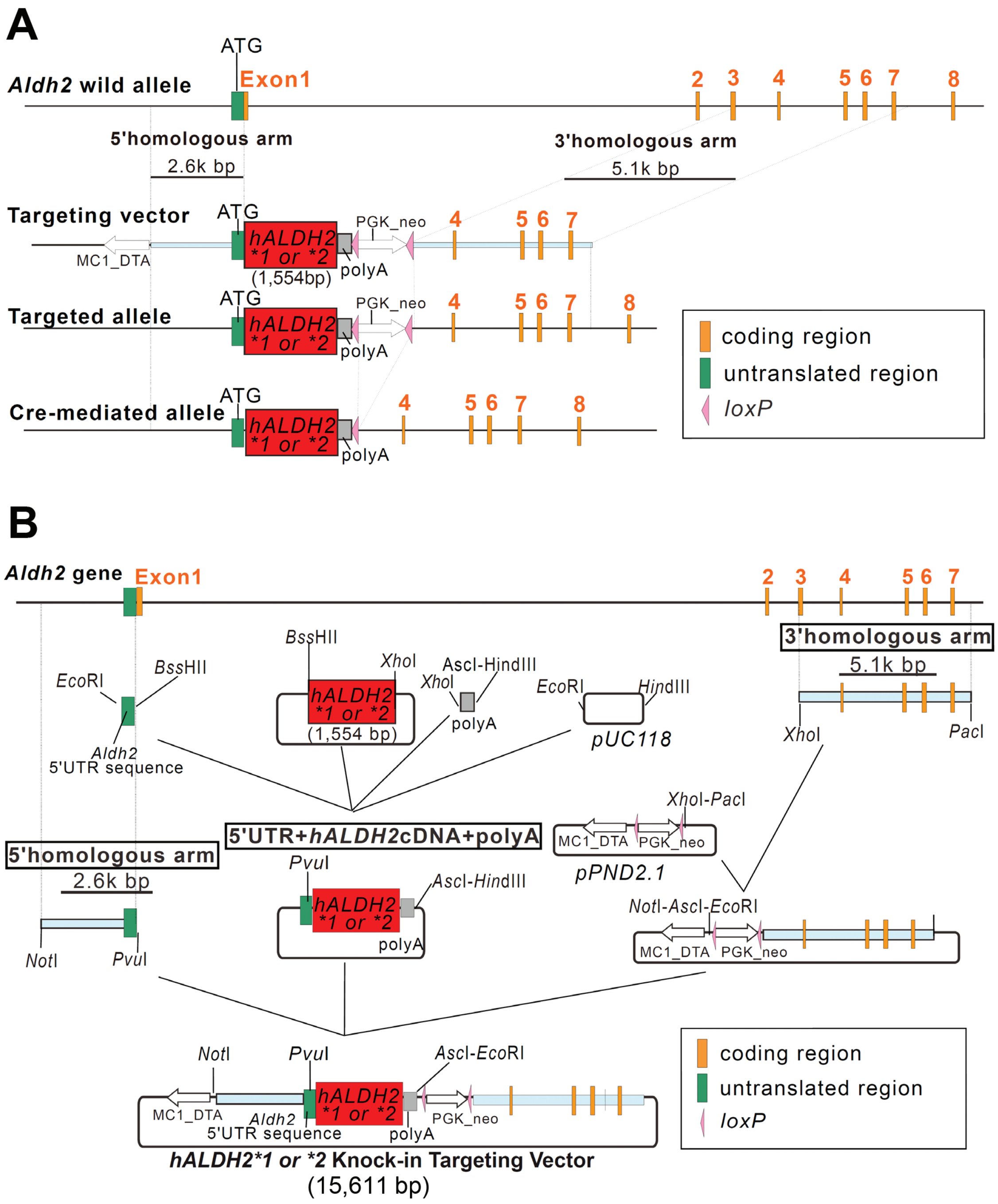

Figure 1. Scheme of human ALDH2*1 or ALDH2*2 targeting vector to generate genetically engineered knock-in mice that express the human ALDH2*1 or ALDH2*2 gene. (**A**) Human *ALDH2* (ALDH2*1 or *ALDH2*) cDNA knock-in strategy. bp: base pair. (**B**) Construction for human ALDH2*1 or ALDH2*2 knock-in targeting vector. 5′ homologous arm (2.6k bp), 5′ UTR of exon 1 of Aldh2 gene + human *ALDH2* (ALDH2*1 or *ALDH2*) cDNA (1554 bp) + polyA containing vector, and 3′ homologous arm (5.1k bp) are included in human ALDH2*1 or ALDH2*2 knock-in targeting vector.

mice (CAG-Cre mice), which was provided by the RIKEN BRC (Tsukuba, Japan), to eliminate neomycin-resistant gene. Consequently, human ALDH2*1/*1 or ALDH2*2/*2 knock-in mice were generated. And then, the human ALDH2*1/*1 and ALDH2*2/*2 knock-in mice were crossed to generate human ALDH2*1/*2 knock-in mice.

The genotype of Aldh2 mice and human *ALDH2* mice was confirmed by PCR amplification using DNA extracted from mouse tails. The primers used to identify mouse *Aldh2* were as follows: forward, 5′-GAGGACTGTGTTGGGAGGTC-3′; reverse, 5′-GTAGGTCCGGTCCCGTTC-3′ (264 bp fragment). The PCR conditions used were 94°C for 3 min, followed by 30 cycles of 94°C for 30 s, 62°C for 30 s and 72°C for 30 s. A final extension was conducted at 72°C for 3 min. The primers used for the detection of human *ALDH2* were as follows: forward, 5′-AGATGTGCAGGATGGCATGACC-3′; reverse, 5′-ACCCGGTAGAATTTCGACGACC-3′ (790 bp fragment). The PCR conditions used were 94°C for 3 min, followed by 30 cycles of 98°C for 10 s, 62°C for 30 s and 68°C for 1 min. A final extension was conducted at 68°C for 3 min. To confirm the elimination of the neomycin-resistance gene, we used the following primers: forward, 5′-AGATGTGCAGGATGGCATGACC-3′; reverse, 5′-GGATGCTATGAGCTTCATTC-3′ (748 bp fragment). The PCR conditions used were 94°C for 3 min, followed by 30 cycles of 98°C for 10 s, 62°C for 30 s and 68°C for 2 min. A final extension was conducted at 68°C for 3 min.

Table 1. Primer sets for amplification from genomic DNA

	Primer name	Sequence(5´-3´)	Size (mer)
PCR for 5´ homologous arm			
Forward	5AF1	CAC TAT CTT CTT CTT CCC TGT GC	23
Reverse	5AR1	GAA GTG AGT CTC ACC TGG TTG C	22
PCR for 3´ homologous arm			
Forward	3AF1	TGA ATG AAG CTC ATA GCA TCC	21
Reverse	3AR1	GGT CCT TTG TAG GAG GTT ACA GC	23
5´ PCR for screening			
Forward	*sc_5AF2*	CC ATC CAT TCA AGG TAA AGT TCC	23
Reverse	*neo_108r*	CCT CAG AAG AAC TCG TCA AGA AG	23
3´ PCR for screening			
Forward	*neo_MS*	ATT CGC AGC GCA TCG CCT TCT ATC GCC TTC	30
Reverse	*sc_3AR3*	CAG GCA CAG GTT ACT ACT CTT CC	23

In addition, the genotype of human *ALDH2*1* and/or *ALDH2*2* was confirmed by the PCR-restriction fragment length polymorphism (RFLP) methods (43). We used the specific restriction enzyme, AcuI (R0641S, New England Biolabs Tokyo, Japan), to distinguish between human *ALDH2*1* and *ALDH2*2*. The PCR conditions for AcuI digestion were 37°C for 90 min, followed by 65°C for 20 min.

Measurement of enzymatic hepatic ALDH2 activity in mice

Hepatic ALDH2 activity in mice was determined as described previously (40). Briefly, it was determined spectrophotometrically by using extracted protein from tissue homogenate by monitoring the reductive reaction of NAD+ to NADH at λ340 nm. All the assays were carried out at 25°C in 0.1 M sodium pyrophosphate buffer, pH = 9.5, 2.4 mM NAD+ and 10 mM acetaldehyde as the substrate.

Measurement of esophageal N^2-ethylidene-dG levels in mice

Esophageal N^2-ethylidene-dG level was quantified as described previously (44). Briefly, DNA was isolated from esophageal tissue specimens. $NaBH_3CN$ (100 mM; 156159, Sigma–Aldrich, St. Louis, MO), a reducing reagent, was added to DNA samples. This converts N^2-ethylidene-dG to stable N^2-Et-dG. As the endogenous N^2-Et-dG level in tissues is extremely low, the N^2-Et-dG level that is converted from N^2-ethylidene-dG indicates the endogenous N^2-ethylidene-dG level (45). The DNA adduct standard, N^2-Et-dG and its stable isotope, [U-15N5]-labeled N^2-Et-dG, were synthesized as described previously (46). DNA samples were digested as described previously (46) and subjected to liquid chromatography-tandem mass spectrometry (LC/MS/MS). LC/MS/MS analyses were performed using a Shimadzu LC system (Shimadzu Corp., Kyoto, Japan) interfaced with a Quattro Ultimo triple-stage quadrupole mass spectrometer or an ACQUITY UPLC H-Class system interfaced with a XEVO-TSQ triple-stage quadrupole mass spectrometer (Waters Corp., Milford, MA), as reported previously (46). Shim-pack XR-ODS columns (3.0 × 75 mm, 2.2 μm; Shimadzu Corp.) or ACQUITY UPLC BEH C18 columns (2.1 × 100 mm, 1.7 μm; Waters Corp.) were used to separate the samples.

Single injection of Alda-1, an ALDH2 activator, on *ALDH2* knock-in mice

To examine the effects of Alda-1 on ALDH2 activation, we injected Alda-1 (20 mg/kg body weight; SML-0462, Sigma–Aldrich) or vehicle control (dimethyl sulfoxide, 472301, Sigma–Aldrich) intraperitoneally on *ALDH2*1/*1*, *ALDH2*1/*2* and/or *ALDH2*2/*2* knock-in mice. Liver of those mice was collected 3 h after the injection and used for measuring the hepatic ALDH activity.

Alda-1 treatment in *ALDH2* knock-in mice with alcohol drinking

*ALDH2*1/*1*, *ALDH2*1/*2* and *ALDH2*2/*2* knock-in mice were given 10% ethanol instead of water and allowed to drink freely and injected intraperitoneally with either Alda-1 (20 mg/kg body weight twice a day, $n = 8$) or vehicle control (dimethyl sulfoxide, $n = 8$) every 12 h for 7 days. And the esophageal tissues were collected for measuring N^2-ethylidene-dG levels.

Single injection of cyanamide, an ALDH2-inhibitor, on C57BL/6N mice

Cyanamide (187364, Sigma–Aldrich), a well-established ALDH2-inhibitor (47), was dissolved in distilled water. We injected cyanamide (1.5 mg/kg body weight) or distilled water intraperitoneally in male C57BL/6N mice, and then hepatic ALDH activity was measured at 2 h after the injection.

Cyanamide treatment on alcohol drinking in C57BL6 mice

Male C57BL/6N mice were allowed to drink 10% ethanol freely and were injected intraperitoneally with either cyanamide (1.5 mg/kg body weight, twice a day, $n = 6$) or vehicle control (distilled water, $n = 6$) every 12 h for 7 days, and the esophageal tissues were collected to measure N^2-ethylidene-dG level.

TaqMan gene expression assays

RNA was isolated using RNeasy Plus Mini Kits (QIAGEN, Hilden, Germany) and cDNA was synthesized using PrimeScript RT reagent kits (Takara Bio, Kusatsu, Japan) according to the manufacturer's instructions. Real-time reverse-transcription PCR was conducted with TaqMan Gene Expression Assays (Life Technologies Corp., Tokyo, Japan) for *ALDH2* (Assay ID; Hs01007998_m1) and for β-actin (Assay ID; Hs99999903_m1) using a LightCycler 480 Instrument II (Hoffmann-La Roche Ltd., Basel, Switzerland) as described previously (48). All PCRs were performed in triplicate. The relative *ALDH2* messenger RNA expression level was normalized to that of β-actin as an internal control.

Western blot analysis

Hepatic whole-cell lysates were prepared as described previuosly (49). The denatured protein samples (20 μg) were fractionated on Any kD Mini-PROTEAN TGX Precast Gels (Bio-Rad Laboratories, Hercules, CA). Primary antibodies and the titers used for western blotting were as follows: goat polyclonal anti-ALDH2 (N-14) (sc-48838; Santa Cruz Biotechnology, Santa Cruz, CA;1:1000); rabbit monoclonal anti-β-actin (13E5; Cell Signaling Technology; 1:2000). These were then reacted with the appropriate horseradish peroxidase-conjugated secondary antibody (GE Healthcare, Little Chalfont, UK). The signal was visualized using an Immobilon Western Chemiluminescent Horseradish Peroxidase Substrate (Merck Millipore, Darmstadt, Germany) and was exposed using a ChemiDoc XRS system equipped with Image Lab software (Bio-Rad Laboratories).

Statistical analysis

Data are presented as the mean ± standard deviation. The data were first tested for normality of distribution. The differences between two groups were analyzed using two-tailed paired Student's *t*-tests for equal variance data. *P*-values of less than 0.05 were considered significant.

Results

Generation of human _ALDH2*1/*1, ALDH2*1/*2,_ or _ALDH2*2/*2_ knock-in mice

To confirm the generation of human _ALDH2_ (_ALDH2*1_ or _ALDH2*2_) gene knock-in mice, we first checked the deletion of mice _Aldh2_ gene and insertion of human _ALDH2_ gene by PCR using the primers specifically recognizing mice _Aldh2_ or human _ALDH2_. As shown in Figure 2A, control C57BL/6N mice (mice/mice) were positive for mice _Aldh2_ and negative for human _ALDH2_. F1 heterozygous human _ALDH2_ knock-in mice (mice _Aldh2_/human _ALDH2*1_ or _ALDH2*2_) showed positive for both mice _Aldh2_ and human _ALDH2_. F2 homozygous human _ALDH2_ knock-in mice (human _ALDH2*1/*1_ or _ALDH2*2/*2_) showed negative for mice _Aldh2_ and positive for human _ALDH2_.

Next, we eliminated neomycin-resistant gene in F2 homozygous human _ALDH2*1/*1_ or _ALDH2*2/*2_ knock-in mice by crossed with CAG-Cre mice, and its absence was confirmed by PCR. When the mice possess the neomycin-resistant gene, 2498 bp bands are seen. On the other hand, 748 bp bands are observed when neomycin-resistant gene is deleted (Figure 2B).

Next, in F2 homozygous human _ALDH2_ knock-in mice, we checked the genotype (i.e. either _ALDH2*1/*1, ALDH2*1/*2_ or _ALDH2*2/*2_) by PCR-RFLP method. After digestion by restriction enzyme AcuI, PCR products derived from human _ALDH2*1/*1_ knock-in mice were recognized by showing three bands at 430 bp, 235 bp and 83 bp, whereas those from human _ALDH2*2/*2_ knock-in mice presented with two bands at 665 bp and 83 bp. After cleavage by AcuI, PCR products derived from human _ALDH2*1/*2_ knock-in mice showed four bands at 665 bp, 430 bp, 235 bp and 83 bp, and thus, human _ALDH2*1/*1_, _ALDH2*1/*2_ and _ALDH2*2/*2_ knock-in mice were generated, and hereafter, those mice are described as _ALDH2*1/*1_, _ALDH2*1/*2_ and _ALDH2*2/*2_ mice, respectively.

Characterization of _ALDH2*1/*1_, _ALDH2*1/*2_ and _ALDH2*1/*2_ mice

We then characterized _ALDH2*1/*1_, _ALDH2*1/*2_ and _ALDH2*2/*2_ mice, in terms of expression and enzymatic activity of ALDH2. When measured using liver tissue homogenate, the _ALDH2_ messenger RNA (Figure 3A) and ALDH2 protein (Figure 3B) expression levels did not differ among three groups. In contrast, ALDH activity in _ALDH2*1/*2_ and _ALDH2*2/*2_ mice was significantly lower than that in _ALDH2*1/*1_ mice (Figure 3C).

Next, we investigated the effect of _ALDH2_ genotype in mice on alcohol consumption as well as alcohol-derived DNA damage in esophageal tissues. When mice were given 10% ethanol as substitute for water for 7 days, the average amount of ethanol consumption in _ALDH2*1/*2_ or _ALDH2*2/*2_ mice was significantly less than that in _ALDH2*1/*1_ mice (Figure 4A). Alcohol drinking increased esophageal DNA damage represented by N^2-ethylidene-dG levels in all groups. Of note, _ALDH2*1/*2_ and _ALDH2*2/*2_ mice showed significantly higher DNA damage than _ALDH2*1/*1_ mice (Figure 4B).

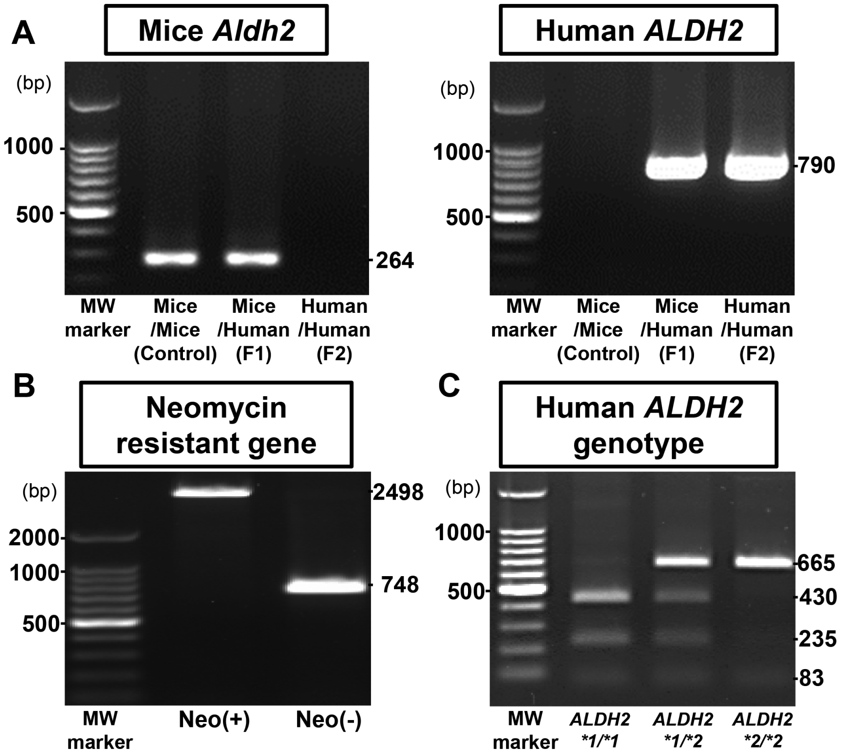

Figure 2. Genotyping of human _ALDH2*1/*1, ALDH2*1/*2 and ALDH2*2/*2_ knock-in mice. (**A**) PCR images for mice _Aldh2_ (left panel), and human _ALDH2_ (right panel). Left lane in left and right panel: molecular weight (MW) marker, 100 base pair (bp) DNA ladder. PCR products of mice _Aldh2_ and human _ALDH2_ were 264 bp and 790 bp, respectively. (**B**) Elimination of neomycin resistant gene. Left lane: MW marker (100 bp DNA ladder). PCR products of human _ALDH2_ with or without neomycin-resistant gene were 2498 bp and 748 bp, respectively. Neo (+): before elimination of neomycin resistant gene, Neo (–): after elimination of neomycin resistant gene. (**C**) The PCR images (PCR-RFLP methods) for _ALDH2*1/*1_, _ALDH2*1/*2_ and _ALDH2*2/*2 mice_. Left lane: MW marker (100 bp DNA ladder). Digestion of PCR products with AcuI harbored three bands (430, 235 and 83 bp) in _ALDH2*1/*1_ mice, four bands (665, 430, 235 and 83 bp) in _ALDH2*1/*2_ mice and two bands (665 and 83 bp) in _ALDH2*2/*2_ mice.

Alda-1 treatment on ALDH2*1/*1, ALDH2*1/*2 and ALDH2*2/*2 mice

To investigate the effects of Alda-1, an ALDH2 activator, we intraperitoneally injected Alda-1 in ALDH2*1/*1, ALDH2*1/*2 and ALDH2*2/*2 mice. As shown in Figure 5A, Alda-1 significantly elevated hepatic ALDH activity in all the human *ALDH2* knock-in mice. The amount of alcohol drinking in ALDH2*1/*2 and ALDH2*2/*2 mice was significantly increased by Alda-1 treatment, but not in ALDH2*1/*1 mice (Figure 5B). Importantly, esophageal N^2-ethylidene-dG level in both ALDH2*1/*2 and ALDH2*2/*2 mice with alcohol drinking were significantly reduced by Alda-1 treatment despite the increased alcohol consumption, indicating the protective role of Alda-1 from alcohol-derived DNA damage in these mice (Figure 5C).

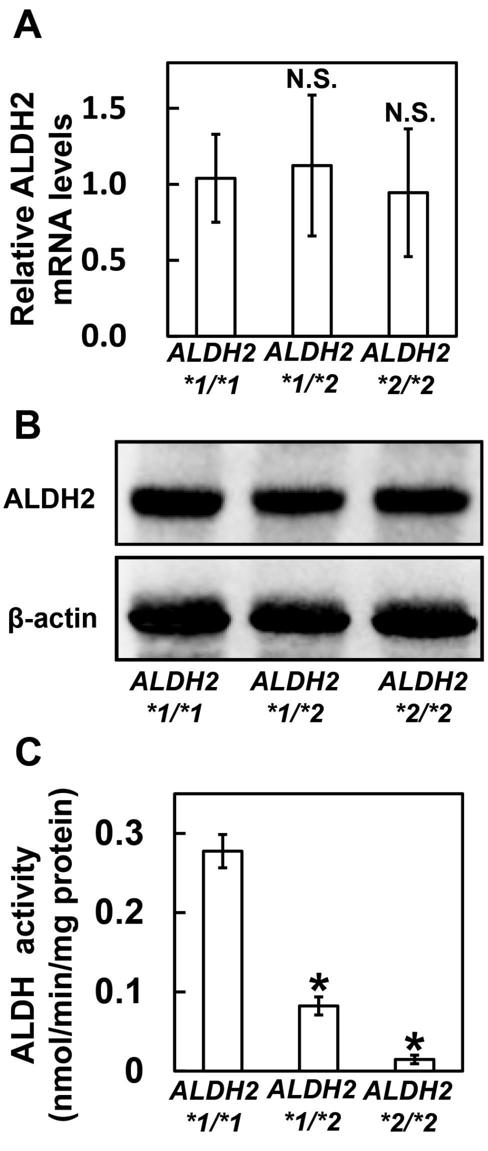

Figure 3. Levels of ALDH2 expression and activity in human ALDH2*1/*1, ALDH2*1/*2 and ALDH2*2/*2 knock-in mice. (**A**) Hepatic *ALDH2* messenger RNA (mRNA) expression levels in ALDH2*1/*1, ALDH2*1/*2 and ALDH2*2/*2 mice: *ALDH2* mRNA expression level was measured by real-time RT-PCR. Relative *ALDH2* mRNA levels in the liver of ALDH2*1/*2 and ALDH2*2/*2 mice relative to that of ALDH2*1/*1 mice is indicated. β-actin served as an internal control. (NS, not significant versus ALDH2*1/*1; $n = 3$). (**B**) Hepatic ALDH2 protein expression in human ALDH2*1/*1, ALDH2*1/*2 and ALDH2*2/*2 knock-in mice: ALDH2 protein expression was demonstrated by western blotting. β-actin served as a loading control. (**C**) Hepatic ALDH activity in ALDH2*1/*1, ALDH2*1/*2 and ALDH2*2/*2 mice. ALDH activity in mice with ALDH2*2 allele (ALDH2*1/*2 and/or ALDH2*2/*2 mice) were significantly lower than that in ALDH2*1/*1 mice. $n = 6$ $^*P < 0.05$ versus ALDH2*1/*1 mice.

Effects of cyanamide, an ALDH2 inhibitor, on esophageal DNA damage in mice with alcohol drinking

Finally, we investigated whether cyanamide, an ALDH2 inhibitor, enhances alcohol-derived DNA damage in the esophagus of mice with alcohol drinking. As shown in Figure 6A, single intraperitoneal injection of cyanamide in C57BL/6N mice significantly reduced hepatic ALDH activity. Thereafter, we injected cyanamide to the C57BL/6N mice and let them drink 10% ethanol for 7 days. Cyanamide treatment resulted in a significant reduction of alcohol consumption (Figure 6B); however, esophageal N^2-ethylidene-dG level was conversely significantly exacerbated by cyanamide treatment (Figure 6C).

Discussion

In this study, we established novel genetic engineering mouse, human ALDH2*1/*1, ALDH2*1/*2 and ALDH2*2/*2 knock-in mice. So far, *Aldh2* knockout mice have been widely used as an experimental mouse model for analyzing Aldh2 function; however, because ALDH2 dysfunction in human being mainly occurs as a result of having ALDH2*2 allele, *Aldh2* knockout mice do not serve as bona fide model of human diseases related to acetaldehyde. In addition, because *Aldh2* knockout mice lack Aldh2 protein, they cannot be used for examining the effect of ALDH2 enzymatic modulator such as ALDH2 activator and/or ALDH2 inhibitors. Indeed, treatment with ALDH2 activator (Alda-1) or ALDH2 inhibitor (cyanamide) did not affect Aldh2 activity in *Aldh2* knockout mice (data not shown). To overcome disadvantages of *Aldh2* knockout mice, we generated human *ALDH2* knock-in mice. In this study, we newly established mice model, in which mouse *Aldh2* allele was replaced to human ALDH2*1 (wild-type allele) or ALDH2*2 (mutant allele) by homologous recombination, and neomycin-resistant genes are also removed using CAG promoter-mediated Cre-loxP system. Here, we showed that ALDH2 activity in ALDH2*1/*2 and ALDH2*2/*2

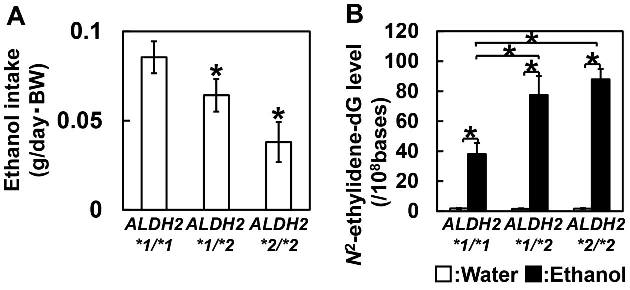

Figure 4. Influences of alcohol drinking on human ALDH2*1/*1, ALDH2*1/*2 and ALDH2*2/*2 knock-in mice. (**A**) The amount of alcohol consumption per day in ALDH2*1/*1, ALDH2*1/*2 and ALDH2*2/*2 mice. They were allowed to drink 10% ethanol freely for 7 days. The average amount of alcohol consumption per day in each mice is shown. $n = 6^*$, $^*P < 0.05$, versus ALDH2*1/*1 mice. (**B**) Esophageal N^2-ethylidene-dG levels in ALDH2*1/*1, ALDH2*1/*2 and ALDH2*2/*2 mice that were allowed to drink 10% ethanol or water for 7 days. Alcohol drinking significantly increased esophageal N^2-ethylidene-dG levels in all groups. Esophageal N^2-ethylidene-dG levels in ALDH2*1/*2 and/or ALDH2*2/*2 mice with alcohol drinking were significantly higher than those in ALDH2*1/*1 mice with alcohol drinking. $n = 6$, $^*P < 0.05$ versus each group of mice that drink water. $^*P < 0.05$ versus ALDH2*1/*1 mice with alcohol drinking.

mice was significantly lower than that in *ALDH2*1/*1* mice, although the messenger RNA as well as protein expression levels of ALDH2 did not differ among them. Therefore, it is conceivable that reduced ALDH2 activity in *ALDH2*1/*2* and/or *ALDH2*2/*2* mice is caused by genetic polymorphism, but not by different expression levels of ALDH2. Notably, even with lesser amount of ethanol intake, esophageal DNA damage levels with alcohol drinking were significantly higher in *ALDH2*1/*2* and *ALDH2*2/*2* mice than those in *ALDH2*1/*1* mice. This result suggests that esophagus of *ALDH2*1/*2* and/or *ALDH2*2/*2* mice is more susceptible to ethanol, probably due to decreased activity of ALDH2. Taken together, we established experimental mice model that possess identical polymorphism seen in human *ALDH2* gene and would serve as an ideal experimental model for studying human diseases associated with impaired ALDH2 activity.

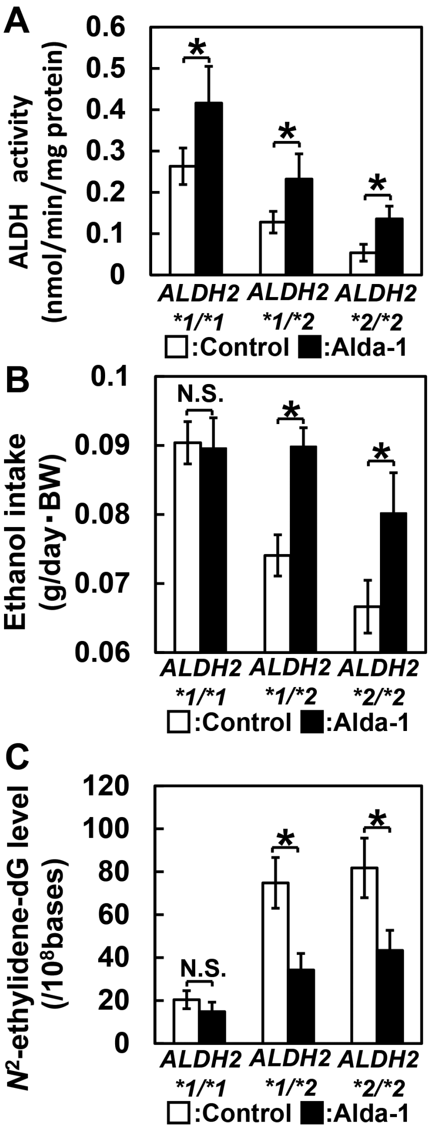

Figure 5. Effect of Alda-1 treatment on human *ALDH2*1/*1*, *ALDH2*1/*2* and *ALDH2*2/*2* knock-in mice. (**A**) Hepatic ALDH activity in *ALDH2*1/*1*, *ALDH2*1/*2* and *ALDH2*2/*2* mice treated with Alda-1 or vehicle control. Alda-1 or vehicle control (20 mg/kg body weight) was intraperitoneally injected to *ALDH2*1/*1*, *ALDH2*1/*2* and *ALDH2*2/*2* mice, and then liver of those mice was collected 3 h after the injection and hepatic ALDH activity was measured. Alda-1 significantly increased hepatic ALDH activity in *ALDH2*1/*1*, *ALDH2*1/*2* and *ALDH2*2/*2* mice. $n = 8$, $^*P < 0.05$ versus each group of mice treated with vehicle control. (**B**) Amount of ethanol intake in *ALDH2*1/*1*, *ALDH2*1/*2* and *ALDH2*2/*2* mice treated with Alda-1 or vehicle control. Alda-1 significantly increased the amount of alcohol drinking per day in both *ALDH2*1/*2* and *ALDH2*2/*2* mice. $n = 8$, $^*P < 0.05$ versus each group of mice treated with vehicle control. N.S.: no significant. (**C**) Esophageal N^2-ethylidene-dG levels in *ALDH2*1/*1*, *ALDH2*1/*2* and *ALDH2*2/*2* mice with alcohol drinking in the presence or absence of Alda-1 treatment. Alda-1 treatment resulted in a significant reduction of the N^2-ethylidene-dG level in the esophagus of both *ALDH2*1/*2* and *ALDH2*2/*2* mice with alcohol drinking. $n = 8$, $^*P < 0.05$ versus each group of mice treated with vehicle control. N.S.: no significant.

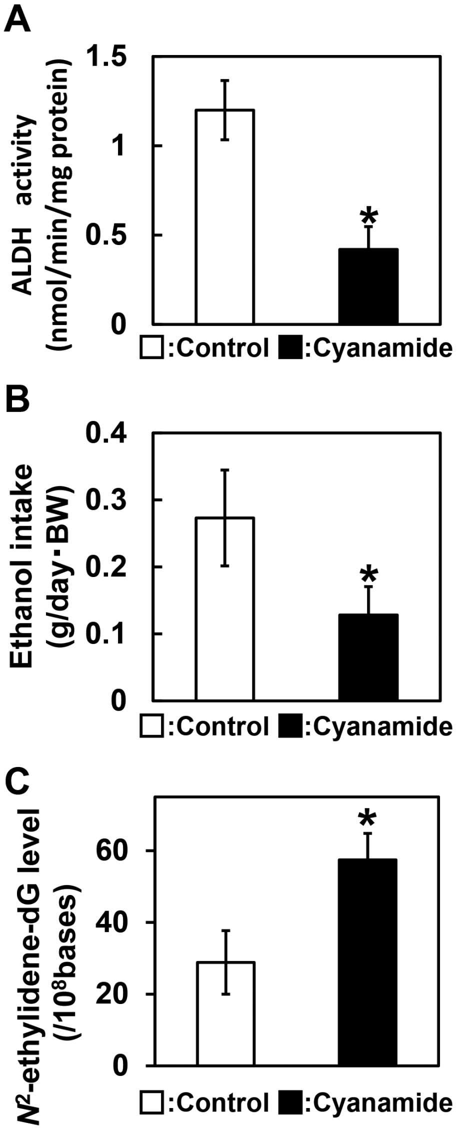

Figure 6. Effect of cyanamide treatment on C57BL6 mice. (**A**) Hepatic ALDH activity in C57BL/6N mice treated with cyanamide or vehicle control. Cyanamide or vehicle control (1.5 mg/kg body weight) was intraperitoneally injected to C57BL6 mice, and then liver of those mice was collected 3 h after the injection and hepatic ALDH activity was measured. $n = 6$, $^*P < 0.05$ versus control. (**B**) The amount of alcohol consumption per day in C57BL/6N mice treated with cyanamide or vehicle control. They were allowed to drink 10% ethanol freely for 7 days. The average amount of alcohol consumption per day in each mice is shown. $n = 6$, $^*P < 0.05$ versus control. (**C**) Esophageal N^2-ethylidene-dG levels in C57BL/6N mice with alcohol drinking in the presence or absence of cyanamide treatment. Cyanamide treatment significantly increased esophageal N^2-ethylidene-dG level in C57BL/6N mice with alcohol drinking, compared with control. $n = 6$, $^*P < 0.05$ versus control.

In this study, we used Alda-1, a small molecule compound that has been identified as ALDH2 activators, and examined its effects on human *ALDH2* knock-in mice. Alda-1 has been firstly reported by Chen *et al.*, identified by high-throughput screening using a fluorescent ALDH2 enzymatic assay based on the emission of resorufin (40). It was shown to be effective for an ischemic damage to the heart (40) and an acute inflammatory pain (50) in human *ALDH2*1/*2* knock-in mice established in their group. In our present study, Alda-1 treatment significantly restored ALDH2 activity in *ALDH2*1/*2* and *ALDH2*2/*2* mice. It also increased the amount of alcohol drinking in those mice, nevertheless, esophageal DNA damage levels were significantly reduced by Alda-1 treatment, presumably due to the increased ALDH2 activity by Alda-1 treatment. Conversely, treatment with cyanamide, an ALDH2 inhibitor, reduced ALDH2 activity in C57BL/6N mice. Despite that cyanamide decreased the alcohol consumption in those mice, esophageal DNA damage levels were significantly enhanced by cyanamide treatment. These results indicate that modulation of ALDH2 activity using ALDH2 activator (Alda-1) or ALDH2 inhibitor (cyanamide) deeply affects the levels of esophageal DNA damage associated with alcohol drinking. Moreover, because esophageal DNA damage has been linked to esophageal carcinogenesis (51), Alda-1 might be effective in the prevention of alcohol-mediated ESCC via restoration of ALDH2 activity.

Molecular mechanisms underlying the activation of ALDH2 by Alda-1 is previously reported by Perez-Miller *et al.*, and they noted that Alda-1 increases ALDH2 enzymatic activity of *ALDH2*2* because it acts as a structural chaperon and it restores the abnormal structure of *ALDH2*2* (41). Furthermore, Alda-1 activates both ALDH2*1 and ALDH2*2 by binding at the entrance of the catalytic tunnel in close proximity to Cys302 and Glu286, which are critical to its substrate catalysis (41). In line with their report, we showed that Alda-1 treatment increased ALDH2 activity not only in *ALDH2*1/*2* and *ALDH2*2/*2* but also in *ALDH2*1/*1* mice. Our results were consistent with a previous report that Alda-1 increased ALDH2 activity in human *ALDH2*1/*1*, *ALDH2*1/*2* and *ALDH2*2/*2* knock-in mice that were established by other groups independently (50). Although Alda-1 did not affect alcohol consumption as well as DNA adduct level in *ALDH2*1/*1* mice, we presume that basal ALDH2 activity in wild-type *ALDH2* might be inherently sufficient, and the increase of ALDH2 activity by Alda-1 in *ALDH2*1/*1* may not have substantial benefit for protecting esophageal DNA damage.

In this study, we did not find histological abnormalities in the esophagus of *ALDH2*1/*2* and *ALDH2*2/*2* mice after drinking alcohol for 7 days. Although we made *ALDH2*1/*2* mice drink 10% alcohol for as long as 12 months, even those mice did not develop esophageal cancer (data not shown). We suspect that more long-time drinking may be necessary for a development of ESCC, or other factors besides alcohol drinking alcohol may also be involved in the development of esophageal cancer, as there has been a report that esophageal dysplasia was caused by 4-nitroquinoline-1-oxide and ethanol intake for 10 weeks (52). Furthermore, we may be able to test the preventive effect of Alda-1 on alcohol-mediated ESCC development with those models.

The limitation of our study is that we could not evaluate the influences of Alda-1 on alcohol-abuse. As cyanamide is used as an alcohol deterrent drug for alcoholics, ALDH2 activators may have the risk of alcohol abuse due to enabling more alcohol drinking and therefore alcohol abuse. We should further define the indication for treatment of ALDH2 activators as a preventive agent for ESCC.

In conclusion, we established a novel mice model representing human *ALDH2* gene polymorphism which causes reduced ALDH2 activity and is associated with various diseases including ESCC. We further showed that ALDH2 activation by Alda-1 in mice with ALDH2 dysfunction alleviates esophageal DNA damages associated with alcohol drinking. Our findings provide a new insight into alcohol-mediated esophageal carcinogenesis and prevention.

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