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Original Article

Impairment of aldehyde dehydrogenase 2 increases accumulation of acetaldehyde-derived DNA damage in the esophagus after ethanol ingestion

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Abstract: Ethanol and its metabolite, acetaldehyde, are the definite carcinogens for esophageal squamous cell carcinoma (ESCC), and reduced catalytic activity of aldehyde dehydrogenase 2 (ALDH2), which detoxifies acetaldehyde, increases the risk for ESCC. However, it remains unknown whether the *ALDH2* genotype influences the level of acetaldehyde-derived DNA damage in the esophagus after ethanol ingestion. In the present study, we administered ethanol orally or intraperitoneally to *Aldh2*-knockout and control mice, and we quantified the level of acetaldehyde-derived DNA damage, especially N^2-ethylidene-2'-deoxyguanosine (N^2-ethylidene-dG), in the esophagus. In the model of oral ethanol administration, the esophageal N^2-ethylidene-dG level was significantly higher in *Aldh2*-knockout mice compared with control mice. Similarly, in the model of intraperitoneal ethanol administration, in which the esophagus is not exposed directly to the alcohol solution, the esophageal N^2-ethylidene-dG level was also elevated in *Aldh2*-knockout mice. This result indicates that circulating ethanol-derived acetaldehyde causes esophageal DNA damage, and that the extent of damage is influenced by knockout of *Aldh2*. Taken together, our findings strongly suggest the importance of acetaldehyde-derived DNA damage which is induced in the esophagus of individuals with *ALDH2* gene impairment. This provides a physiological basis for understanding alcohol-related esophageal carcinogenesis.

Keywords: Carcinogenesis, esophageal squamous cell carcinoma, acetaldehyde, acetaldehyde-derived DNA damage, DNA adduct

Introduction

Squamous cell carcinoma (SCC) is the predominant histological type of esophageal cancer worldwide, particularly in east Asian countries [1]. Epidemiological studies have clearly shown that chronic ethanol consumption and acetaldehyde produced from ethanol contained in alcoholic beverages increase the risk of cancers including esophageal SCC (ESCC) [2]. The International Agency for Research on Cancer certified acetaldehyde from consuming alcoholic beverages as 'the group I carcinogens' [3]. Ethanol is absorbed mainly from the duodenum and jejunum, and is transported to the liver, where it is metabolized to acetaldehyde by alcohol dehydrogenase, and acetaldehyde is subsequently detoxified to acetic acid by aldehyde dehydrogenase 2 (ALDH2) [4]. Heavy alcohol consumers with the inactive *ALDH2* genotype are reported to have a greater risk for ESCC [5-7]. Thus, reduced catalytic activity of ALDH2 is considered to play crucial roles in the development of ESCC [1, 5, 8].

Acetaldehyde is a highly reactive compound that can interact with DNA to form DNA adducts, which induce DNA mutations [9-13]. Previous

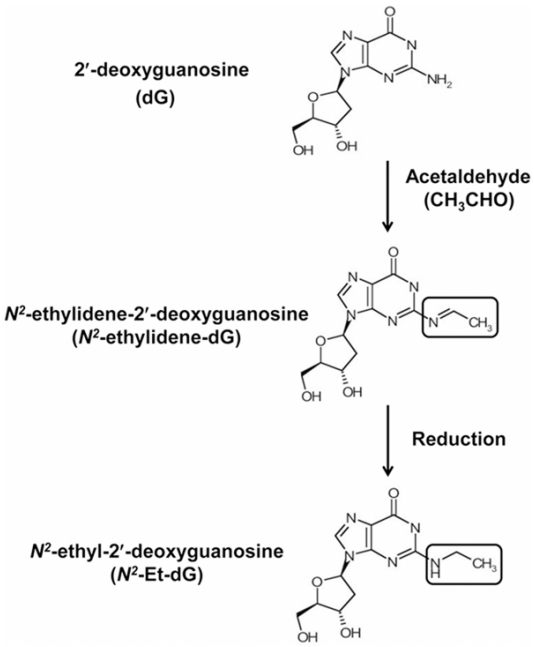

Figure 1. Scheme of the formation of acetaldehyde-derived DNA adducts. Acetaldehyde binds to 2'-deoxyguanosine (dG) and forms N^2-ethylidene-2'-deoxyguanosine (N^2-ethylidene-dG). N^2-ethyl-2'-deoxyguanosine (N^2-Et-dG) is generated by reduction of N^2-ethylidene-dG.

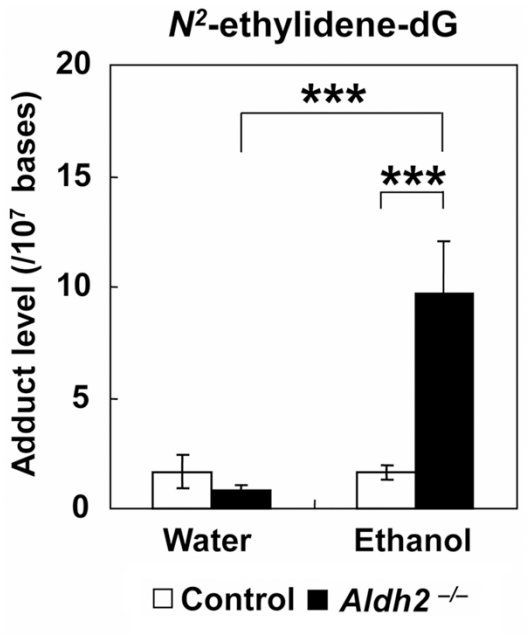

Figure 2. N^2-ethylidene-dG levels after ethanol consumption in *Aldh2*$^{-/-}$ mice. *Aldh2*$^{-/-}$ and control mice were either allowed to drink 5% ethanol or were given water for 8 weeks, and the esophageal N^2-ethylidene-dG level was quantified. The N^2-ethylidene-dG level was significantly higher in the esophagus of ethanol-drinking *Aldh2*$^{-/-}$ mice compared with ethanol-drinking control mice (***$P < 0.001$) and with water-drinking *Aldh2*$^{-/-}$ mice (***$P < 0.001$) (n = 5 in each group).

reports have shown that there are several kinds of acetaldehyde-derived DNA adducts including N^2-ethylidene-2'-deoxyguanosine (N^2-ethylidene-dG), N^2-ethyl-2'-deoxyguanosine (N^2-Et-dG), and 1',N^2-propano-2'-deoxyguanosine [14-16]. Among them, N^2-ethylidene-dG is the most abundant DNA adduct derived from acetaldehyde [15, 17]. Matsuda et al. reported that the N^2-ethylidene-dG level in the liver or stomach is elevated by ethanol consumption in experimental mouse models [16, 17]. Thus, quantification of acetaldehyde-derived DNA adducts provide an index of direct DNA damage caused by acetaldehyde [15-18].

Since the report by Slaughter et al. in 1953, the multicentric development of SCC has been recognized in the squamous epithelium of the esophagus as well as in the head and neck region. Such development is termed 'field cancerization' [19], and its occurrence is closely associated with alcohol consumption and *ALDH2* gene polymorphism [20-22]. It has been suggested that genetic damage induced by acetaldehyde accumulates in the esophageal mucosa and that this damage is involved in the multicentric development of SCC. However, it remains unclear how alcohol consumption and impairment of *ALDH2* promote ESCC development.

To examine whether the *ALDH2* genotype determines the level of acetaldehyde-derived DNA damage in the esophagus associated with ethanol consumption, we administered ethanol orally and intraperitoneally in *Aldh2*-knockout (*Aldh2*$^{-/-}$) mice and quantified the N^2-ethylidene-dG levels in the esophagus.

Materials and methods

Aldh2-knockout mice

Ten-week-old male *Aldh2*$^{-/-}$ mice [23], which had been backcrossed with C57BL/6, were obtained from the Department of Environmental Health, University of Occupational and Environmental Health (Fukuoka, Japan). Control C57BL/6 mice (*Aldh2*$^{+/+}$) were purchased from Charles River Japan (Yokohama, Japan). The genotype of *Aldh2* was determined by polymerase chain reaction as described previously [23, 24].

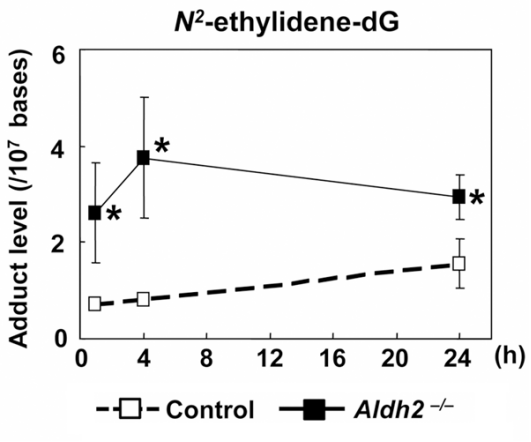

Figure 3. N^2-ethylidene-dG levels after intraperitoneal injection of ethanol in *Aldh2*$^{-/-}$ mice. *Aldh2*$^{-/-}$ and control mice were injected with 1 mL of 5% ethanol intraperitoneally. N^2-ethylidene-dG levels in the esophagus were measured at 1, 4, or 24 h after the injection. The esophageal N^2-ethylidene-dG level was significantly higher in *Aldh2*$^{-/-}$ mice than in control mice at all time points (*P < 0.05 vs. control mice) (n = 3 at each time point).

Free ethanol-drinking model in mice

Aldh2$^{-/-}$ mice and control mice were allowed to drink 5% ethanol or water for 8 weeks (n = 5 per group). The mice were sacrificed, and esophageal tissue specimens were collected, frozen in liquid nitrogen, and stored at -80°C until analyzed. The mice were used in conformity with the regulations of the committee on animal experiments of Kyoto University.

Intraperitoneal ethanol injection model in mice

To examine whether circulating ethanol contributes to induction of DNA damage in the esophagus, we developed an animal model in which *Aldh2*$^{-/-}$ and control mice were injected with 1 mL of 5% ethanol intraperitoneally. The mice were sacrificed at 1, 4, or 24 h after the injection of ethanol (n = 3, at each time point). Esophageal tissues were collected and stored at -80°C. This experiment conformed to the regulations of the committee on animal experiments of the National Cancer Center Hospital East (Kashiwa, Japan).

DNA isolation, digestion, and quantification of N^2-ethylidene-dG

DNA was isolated from tissue specimens using a Gentra Puregene Tissue Kit (Qiagen Inc., Valencia, CA), according to the manufacturer's instructions. We quantified the N^2-ethylidene-dG levels in the esophagus of *Aldh2*$^{-/-}$ and control mice. As shown in **Figure 1**, N^2-ethylidene-dG is the direct DNA adduct derived from acetaldehyde, and N^2-Et-dG is the DNA adduct generated by reduction of N^2-ethylidene-dG. To quantify the N^2-ethylidene-dG level, we used the method of Wang et al. [25]. Briefly, we added reducing agent, $NaBH_3CN$ (final concentration: 100 mM), to the isolated DNA samples. During this procedure, N^2-ethylidene-dG is converted to stable N^2-Et-dG. Because the endogenous N^2-Et-dG level is extremely low, the amount of N^2-Et-dG converted from N^2-ethylidene-dG can be used to estimate the endogenous N^2-ethylidene-dG level [15, 25]. The DNA adduct standard, N^2-Et-dG, and its stable isotope, [U-$^{15}N_5$]-labeled N^2-Et-dG, were synthesized as described previously [16, 26]. Twenty micrograms of DNA sample was digested as described previously [17] and then subjected to liquid chromatography tandem mass spectrometry (LC/MS/MS). LC/MS/MS analyses were performed using a Shimadzu LC system (Shimadzu Corporation, Kyoto, Japan) interfaced with a Quattro Ultimo triple-stage quadruple MS (Waters/Micromass UK Ltd, Manchester, UK) according to the methods as described previously [17].

Statistical analyses

Statistical analyses were performed using SPSS statistics software (version 17; SPSS Inc., Chicago, IL). Data are presented as mean ± standard deviation (SD). The data were analyzed using two-tailed paired t test. P values < 0.05 were considered significant.

Results

N^2-ethylidene-dG level in the esophagus after ethanol consumption in mice

Among the water-drinking groups, the average N^2-ethylidene-dG level in the esophagus was 1.61 ± 0.63 adducts/10^7 bases in control mice and 0.80 ± 0.22 adducts/10^7 bases in *Aldh2*$^{-/-}$ mice. Among the ethanol-drinking groups, the level of N^2-ethylidene-dG was significantly elevated in *Aldh2*$^{-/-}$ mice (9.73 ± 2.33 adducts/10^7 bases) but did not increase in control mice (1.62 ± 0.30 adducts/10^7 bases) (P < 0.001 vs. control mice, n = 5) (**Figure 2**). Ethanol drinking

did not induce obvious histopathological changes in the esophagus of these mice (data not shown).

N^2-ethylidene-dG levels in the esophagus after intraperitoneal injection of ethanol in mice

Intraperitoneal injection of ethanol provides a unique experimental model of alcohol-induced damage because the esophagus is not exposed directly to ethanol. Here, we measured the N^2-ethylidene-dG levels in the esophagus of *Aldh2*$^{-/-}$ and control mice after the intraperitoneal injection of ethanol, and we determined how the *Aldh2* gene influences the induction of DNA damage caused by acetaldehyde derived from circulating ethanol. The N^2-ethylidene-dG levels in the esophagus of control mice at 1, 4, and 24 h after intraperitoneal ethanol injection were 0.71 ± 0.02, 0.79 ± 0.08, and 1.56 ± 0.52 adducts/10^7 bases, respectively. The N^2-ethylidene-dG levels mice were significantly higher in *Aldh2*$^{-/-}$ mice than in control mice at the same time points; the levels in *Aldh2*$^{-/-}$ mice were 2.61 ± 1.05 (P = 0.044), 3.76 ± 1.26 (P = 0.028), 2.93 ± 0.47 (P = 0.014) adducts/10^7 bases (n = 3 at each time point) (**Figure 3**).

Discussion

In this study, we found that impairment of the *Aldh2* gene and ethanol drinking were closely related to the induction of acetaldehyde-derived DNA damage in the esophagus. In our model of intraperitoneal ethanol administration, in which the esophagus is not exposed directly to ethanol, esophageal DNA damage was related to the circulating ethanol-derived acetaldehyde.

Although epidemiological evidence suggests that acetaldehyde is involved in the carcinogenesis of ESCC [5, 20, 21, 27], it is unknown how acetaldehyde acts on the esophagus. In the present study, acetaldehyde-derived genetic damage was assessed by measuring the N^2-ethylidene-dG level in the esophagus. As expected, the esophageal N^2-ethylidene-dG level was significantly increased by ethanol consumption in *Aldh2*$^{-/-}$ mice. This result indicates that the *Aldh2* genotype strongly affects accumulation of acetaldehyde-derived DNA damage in the esophagus after ethanol consumption.

One limitation of the experimental approach using oral ethanol consumption is that one cannot determine whether the DNA adduct level is influenced by the direct exposure of the esophagus to the alcohol solution or by acetaldehyde derived from ethanol circulating systematically after having been absorbed from the gastrointestinal tract. Therefore, we established an experimental mouse model in which the esophagus is not exposed directly to the alcohol solution but, instead, the ethanol is injected into the abdominal cavity. In this model, ethanol is absorbed from the peritoneum and is metabolized to acetaldehyde in the liver, and then acetaldehyde circulates and is distributed systematically. Interestingly, even in this experimental model, the acetaldehyde-derived N^2-ethylidene-dG level was significantly higher in the esophagus of *Aldh2*$^{-/-}$ mice than in control mice. As shown in previous clinical reports, acetaldehyde can be detected in the saliva or exhaled breath after alcohol drinking [28, 29]. In our *in vivo* experiments, we cannot exclude the possibility that the esophagus may have been exposed to acetaldehyde derived from these origins and that this might have affected the N^2-ethylidene-dG level in the esophagus. Regardless, our data provide important evidence that impairment of *ALDH2* is involved in the induction of esophageal DNA adducts caused by acetaldehyde derived from circulating ethanol.

In conclusion, our study strongly suggests the importance of acetaldehyde-derived DNA damage in the alcohol-mediated carcinogenesis of ESCC, especially in individuals with impairment of *ALDH2*. Understanding the mechanisms responsible for this effect may contribute to the development of ways to prevent ESCC.

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Disclosure of conflict of interest

The authors disclose no potential conflicts of interest.

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