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

Yoshiyuki Yukawa¹, Shinya Ohashi²,³, Yusuke Amanuma¹, Yukie Nakai², Mihoko Tsurumaki², Osamu Kikuchi³, Shin’ichi Miyamoto¹, Tsunehiro Oyama⁴, Toshihiro Kawamoto⁴, Tsutomu Chiba¹, Tomonari Matsuda⁵, Manabu Muto²

¹Department of Gastroenterology and Hepatology, Kyoto University Graduate School of Medicine, Kyoto, Japan; ²Department of Therapeutic Oncology, Kyoto University Graduate School of Medicine, Kyoto, Japan; ³Institute for Advancement of Clinical and Translational Science (iACT), Kyoto University Hospital, Kyoto, Japan; ⁴Department of Environmental Health, School of Medicine, University of Occupational and Environmental Health, Fukuoka, Japan; ⁵Research Center for Environmental Quality Management, Kyoto University, Otsu, Japan

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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 N²-ethylidene-2'-deoxyguanosine (N²-ethylidene-dG), in the esophagus. In the model of oral ethanol administration, the esophageal N²-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 N²-ethylidene-dG level was also elevated in Aldh2-knockout mice compared with control 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
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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 $Aldh2$–/– 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].

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**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).
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Free ethanol-drinking model in mice

Aldh2−/− 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 N²-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 N²-ethylidene-dG levels in the esophagus of Aldh2−/− and control mice. As shown in Figure 1, N²-ethylidene-dG is the direct DNA adduct derived from acetaldehyde, and N²-Et-dG is the DNA adduct generated by reduction of N²-ethylidene-dG. To quantify the N²-ethylidene-dG level, we used the method of Wang et al. [25]. Briefly, we added reducing agent, NaBH₃CN (final concentration: 100 mM), to the isolated DNA samples. During this procedure, N²-ethylidene-dG is converted to stable N²-Et-dG. Because the endogenous N²-Et-dG level is extremely low, the amount of N²-Et-dG converted from N²-ethylidene-dG can be used to estimate the endogenous N²-ethylidene-dG level [15, 25]. The DNA adduct standard, N²-Et-dG, and its stable isotope, [U-¹⁵N]labeled N²-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 quadrupole 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

N²-ethylidene-dG level in the esophagus after ethanol consumption in mice

Among the water-drinking groups, the average N²-ethylidene-dG level in the esophagus was 1.61 ± 0.63 adducts/10⁷ bases in control mice and 0.80 ± 0.22 adducts/10⁷ bases in Aldh2−/− mice. Among the ethanol-drinking groups, the level of N²-ethylidene-dG was significantly elevated in Aldh2−/− mice (9.73 ± 2.33 adducts/10⁷ bases) but did not increase in control mice (1.62 ± 0.30 adducts/10⁷ bases) (P < 0.001 vs. control mice, n = 5) (Figure 2). Ethanol drinking
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did not induce obvious histopathological changes in the esophagus of these mice (data not shown).

\[ N^2 \text{-ethylidene-dG} \text{ levels in the esophagus after intraperitoneal injection of ethanol in mice} \]

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 \( \text{Aldh2}^{–/–} \) and control mice after the intraperitoneal injection of ethanol, and we determined how the \( \text{Aldh2} \) 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 \( \text{Aldh2}^{–/–} \) mice than in control mice at the same time points; the levels in \( \text{Aldh2}^{–/–} \) 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 \( \text{Aldh2} \) 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 \( \text{Aldh2}^{–/–} \) mice. This result indicates that the \( \text{Aldh2} \) 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 \( \text{Aldh2}^{–/–} \) 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 \( \text{ALDH2} \) 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 \( \text{ALDH2} \). 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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Address correspondence to: Dr. Manabu Muto, De-
partment of Therapeutic Oncology, Kyoto University
Graduate School of Medicine, 54 Kawahara-cho, Shogoin, Sakyo-ku, Kyoto 606-8507, Japan. Tel:
+81-75-751-4592; Fax: +81-75-751-4594; E-mail:
muto@kuhp.kyoto-u.ac.jp (MM); yoshitak@kuhp.
kyoto-u.ac.jp (YY)
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