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RESEARCH ARTICLE

The effect of ethanol on the formation of N^2-ethylidene-dG adducts in mice: implications for alcohol-related carcinogenicity of the oral cavity and esophagus

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Abstract

The present study aimed to experimentally confirm that long-term alcohol drinking causes a high risk of oral and esophageal cancer in aldehyde dehydrogenase 2 (ALDH2)-deficient individuals. *Aldh2* knockout mice, an animal model of ALDH2-deficiency, were treated with 8% ethanol for 14 months. Levels of acetaldehyde-derived DNA adducts were increased in esophagus, tongue and submandibular gland. Our finding that a lack of Aldh2 leads to more DNA damage after chronic ethanol treatment in mice supports epidemiological findings on the carcinogenicity of alcohol in ALDH2-deficient individuals who drink chronically.

Keywords: Acetaldehyde, ALDH2, carcinogenesis, DNA adduct, ethanol, N^2-ethyl-dG

Introduction

The consumption of all types of alcoholic beverages is associated with an increased cancer risk (IARC, 1988). Numerous studies (Blot 1992; Brooks and Theruvathu 2005; Balbo et al. 2008; Druesne-Pecollo et al. 2009; Yu et al. 2010) suggest that the ethanol metabolic pathway is important to alcohol-related carcinogenicity. When alcoholic beverages are consumed, acetaldehyde is formed from the oxidation of ethanol by alcohol dehydrogenase (ADH), cytochrome P450 2E1 (CYP2E1), and catalase, then subsequently oxidized to form acetate by aldehyde dehydrogenase 2 (ALDH2) (Harada et al. 1980; Brien and Loomis 1983). Acetaldehyde, an intermediate of ethanol metabolism, is a genotoxic compound that causes mutations and sister chromatid exchanges in cultured mammalian cells and also in human lymphocytes (IARC, 1999).

Acetaldehyde is a highly reactive compound that can interact with DNA to form adducts. A trend toward increasing levels of acetaldehyde-derived DNA adducts in the human body with increasing alcohol consumption was reported (Balbo et al. 2008). Scientific studies (Kriek et al. 1993; Pfohl-Leszkowicz et al. 1995; Chen et al. 1999; Saad et al. 2006; Marjani et al. 2010) support that DNA adducts are related to several forms of cancer, for example, colon, bladder, lung, oral and esophageal cancer. Therefore, acetaldehyde-derived DNA adducts may be also important to ethanol-induced carcinogenesis. The major acetaldehyde-derived DNA adduct in the human body is a Schiff base, N^2-ethylidene-2′-deoxyguanosine (N^2-ethylidene-dG) (Vaca et al. 1995). The N^2-ethylidene-dG adduct is considered chemically stable when present in DNA, but unstable when in the single 2′-deoxynucleoside form, with a half-life of just 5 min (Wang et al. 2000). Thus, an analytical approach was developed for quantifying N^2-ethylidene-dG, through the detection of the product of its reduction by $NaBH_3CN$, N^2-ethyl-2′-deoxyguanosine (N^2-ethyl-dG), which is more stable and easier to detect

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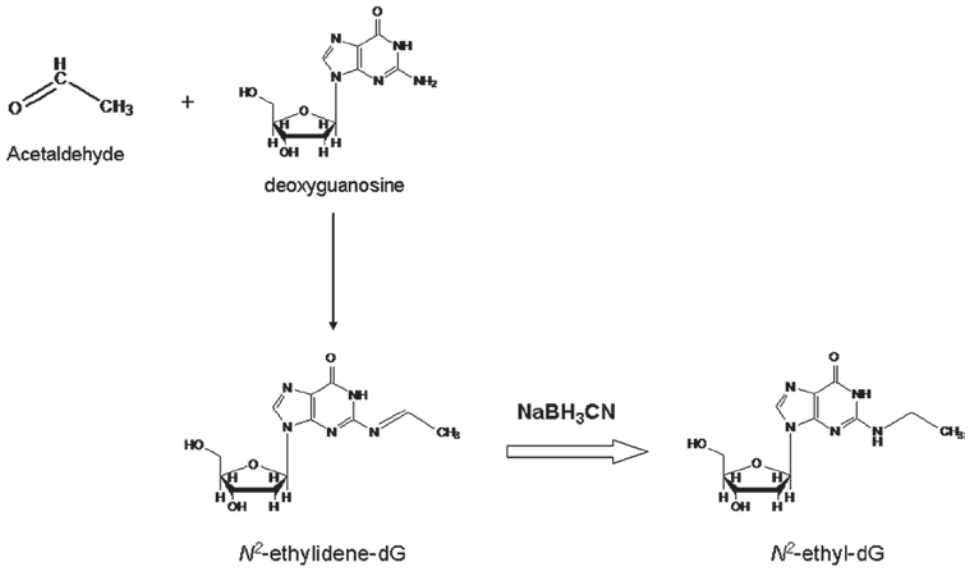

Figure 1. Formation of the N^2-ethylidene-dG and N^2-ethyl-dG adducts. Acetaldehyde can interact with deoxyguanosine to form a Schiff base, N^2-ethylidene-dG. During the reduction step, the unstable N^2-ethylidene-dG was expected to be converted to the stable N^2-ethyl-dG.

(Wang et al. 2006) (Figure 1). Recently, N^2-ethyl-dG has become a potential biomarker for assessing effects of alcohol consumption on DNA, especially for the study of alcohol-related carcinogenicity (Balbo et al. 2008).

A single substitution at nucleotide (nt.) 1510 in the *ALDH2* gene has been characterized as *ALDH2*2* in relation to the wild type, *ALDH2*1*. The enzyme encoded by *ALDH2*2* has a lysine substituted for glutamic acid at residue 487 (Yoshida et al. 1984), resulting in a loss of activity. This inactive allele is observed in Japanese populations with a frequency of around 30% (Kawamoto et al. 1994). *ALDH2*1/*2* heterozygotes have a limited ability (with about 10% residual ALDH2 activity) to metabolize acetaldehyde (Baan et al. 2007). Neither the homozygote (*ALDH2*2/*2*) nor heterozygote (*ALDH2*1/*2*) is able to metabolize acetaldehyde promptly. Thus, both *ALDH2*1/*2* and *ALDH2*2/*2* individuals are characterized as ALDH2-deficient.

Epidemiological studies on the carcinogenicity of alcohol have revealed that the sites most affected are the oral cavity and esophagus, and that drinkers with *ALDH2*1/*2* are more susceptible (Yokoyama and Omori 2003). In contrast, *ALDH2*2/*2* individuals show a lower cancer risk because severe adverse effects keep them away from drinking.

Experimental studies using rodents show that the administration of ethanol causes hepatocellular adenomas and carcinomas, head and neck carcinomas, fore-stomach carcinomas, testicular interstitial-cell adenomas, osteosarcomas of the head, neck, and other sites, and mammary adenocarcinomas (IARC, 2010). However, in these studies, the ethanol was co-administered with known carcinogens.

Therefore, in the present study the authors aimed to experimentally clarify the carcinogenicity of long-term ethanol drinking in relation to Aldh2 activity, without co-administration of carcinogens. *Aldh2* knockout mouse ($Aldh2^{-/-}$ mouse), an animal model of ALDH2-deficiency (*ALDH2*1/*2* individuals), were treated with ethanol for 14 months, and the levels of N^2-ethylidene-dG in the esophagus, tongue and submandibular gland were studied by detecting N^2-ethyl-dG (reduction product of N^2-ethylidene-dG).

Materials and methods

Animals

The generation of $Aldh2^{-/-}$ mice was described previously (Kitagawa et al. 2000). The $Aldh2^{-/-}$ mice were backcrossed with C57BL/6 wild-type mice ($Aldh2^{+/+}$ mice) for more than 10 generations. Three-week-old $Aldh2^{+/+}$ mice and $Aldh2^{-/-}$ mice were used. Both types of mice were kept in an air-conditioned room on a 12-h light/dark cycle with water and food available *ad libitum*. These mice were housed in specific pathogen-free units of the Division of Animal Care at the University of Occupational and Environmental Health (UOEH). All animal treatments were carried out in accordance with the guidelines of the Animal Welfare and Ethics Committee of UOEH (Ethics approval number, AE03-002).

Treatments

Mice were divided into eight groups, that is, male and female $Aldh2^{+/+}$ mice given water, $Aldh2^{-/-}$ mice given water, $Aldh2^{+/+}$mice given 8% ethanol, and $Aldh2^{-/-}$ mice given 8% ethanol. The number of mice in each group is list in Table 1. Mice were given 24h free access to bottles containing water or 8% ethanol in water, and the bottles were changed everyday. After 14 months, the mice were sacrificed to remove tissues. Tissue specimens of the esophagus, tongue and submandibular gland were immediately placed in liquid nitrogen and stored at –80°C.

Table 1. Numbers of mice in each group.

	Water	8% Ethanol
Male		
Aldh2$^{+/+}$ mice	8	8
Aldh2$^{-/-}$ mice	7	8
Female		
Aldh2$^{+/+}$ mice	9	8
Aldh2$^{-/-}$ mice	10	6

Mice received drinking water or 8 % ethanol, and were sacrificed after 14 months of treatment.

DNA isolation

DNA was isolated using a Puregene genomic DNA purification kit (Gentra Systems, Inc., Minneapolis, Minnesota) from 20 to 50 mg of tissue sample as described previously (Matsuda et al. 2007; Oyama et al. 2010). The protocol was modified by adding $NaBH_3CN$ to all solutions (final $NaBH_3CN$ concentration was 100 mM). After the purification step, DNA was dissolved in 10 mM Tris-HCl/5 mM EDTA buffer (pH 7.0), extracted with chloroform, and precipitated with ethanol. Finally, the quantity and quality of DNA were measured using a DU Series 640 Spectrophotometer (Beckman Instruments, Inc., Fullerton, CA) at 260 nm and 280 nm.

DNA digestion

The pretreatment of DNA samples and liquid chromatography tandem mass spectrometry (LC/MS/MS) were carried out as described previously (Matsuda et al. 2006; Matsuda et al. 2007). [$^{15}N_5$] N^2-ethyl-dG was synthesized and used as the internal standard for LC/MS/MS.

Aliquots (20 μg) of DNA were digested into their constituent 2′-deoxyribonucleoside-3′-monophosphate units by the addition of 17 mM citrate plus 8 mM $CaCl_2$ buffer (15 μl) that contained micrococcal nuclease (22.5 U) and spleen phosphodiesterase (0.075 U) plus internal standards. The solutions were mixed and incubated for 3 h at 37°C, after which alkaline phosphatase (1 U), 10 μl of 0.5 M Tris-HCl (pH 8.5), 5 μl of 20 mM $ZnSO_4$, and 67 μl of distilled water were added, and the incubation continued for another 3 h at 37°C. The digested sample was extracted twice with methanol. The methanol fractions were evaporated to dryness, re-suspended in 50 μl of distilled water, and subjected to LC/MS/MS.

Liquid chromatography tandem mass spectrometry

LC/MS/MS was performed using a Shimadzu LC system (Shimadzu, Kyoto, Japan) interfaced with a Quattro Ultima triple stage quadrupole MS (Waters-Micromass, Manchester, UK). The LC column was eluted over a gradient that began at a ratio of 5% methanol to 95% water and was changed to 40% methanol over a period of 30 min, changed to 80% methanol from 30 to 35 min, and finally returned to the original starting conditions, 5:95, for the remaining 11 min. The total run time was 46 min. Sample injection volumes of 20 μl each were separated on a Shim-pack XR-ODS column (3.0 mm × 75 mm, 2.2 μm) and eluted at a flow rate of 0.2 ml/min. Mass spectral analyses were carried out in the positive ion mode with nitrogen as the nebulizing gas. The ion source temperature was 130°C, the desolvation gas temperature was 380°C and the cone voltage was operated at a constant 35 V. Nitrogen gas was also used as the desolvation gas (700 l/h) and cone gas (35 l/h), and argon was used to provide a collision cell pressure of 1.5×10^{-3} mbar. Positive ions were acquired in the multiple reaction monitoring (MRM) mode. The MRM transitions monitored were as follows: [$^{15}N_5$] N^2-ethyl-dG, m/z 301 → 185 and N^2-ethyl-dG, m/z 296 → 180. The amount of each adduct was quantified by the ratio of the peak area of the target adduct to that of its stable isotope. Quanlynx (version 4.0) software (Waters-Micromass) was used to create standard curves and to calculate adduct concentrations. The amount of deoxyguanosine was monitored at 254 nm by a Shimadzu SPD-10A UV-Visible detector that was in place before the tandem MS. The level of N^2-ethyl-dG is shown as N^2-ethyl-dG/10^8 bases.

Statistical analysis

Differences in N^2-ethyl-dG levels between two groups were compared using the Mann–Whitney U test. $P < 0.05$ was considered as statistically significant. All statistical analyses were conducted using SPSS for Windows (version 15.0).

Results

Body weight of mice

During the 14 months of treatment, the mean body weights of water-treated mice before treatment and after treatment were 13.21 ± 3.26 g and 30.05 ± 4.46 g, respectively. For ethanol-treated mice, the mean body weights before and after treatment were 14.38 ± 2.82 g and 30.6 ± 5.5 g, respectively. There were no significant differences between the body weights of water-treated mice and ethanol-treated mice after the 14-month treatment.

N^2-ethyl-dG levels in the esophagus

N^2-ethyl-dG levels in the esophagus of water-treated male and female *Aldh2*$^{+/+}$ mice were 22.7 (15.1–45.4) (median (95% confidence interval)) and 25.3 (15.4–46.3) adducts per 10^8 bases, respectively. In contrast, those of *Aldh2*$^{-/-}$ mice were 57.7 (44.6–70.1) in males and 57.5 (43.2–68.9) in females, 2–2.5 times higher than the median levels in *Aldh2*$^{+/+}$ mice.

Ethanol treatment raised the adduct levels by about 1.3–2-fold in *Aldh2*$^{+/+}$ mice and 1.5–4-fold in *Aldh2*$^{-/-}$ mice. After the 14-month ethanol treatment, the N^2-ethyl-dG levels of *Aldh2*$^{-/-}$ mice were about 2 times higher in males and about 6 times higher in females than those of *Aldh2*$^{+/+}$ mice (Figure 2).

N^2-ethyl-dG levels in the tongue

Median of N^2-ethyl-dG levels in the tongue were around 2 times higher in water-treated *Aldh2*$^{-/-}$ mice (male and female) than water-treated *Aldh2*$^{+/+}$ mice. After 14 months of ethanol treatment, the N^2-ethyl-dG levels of *Aldh2*$^{-/-}$ mice were about 2 times higher in males and

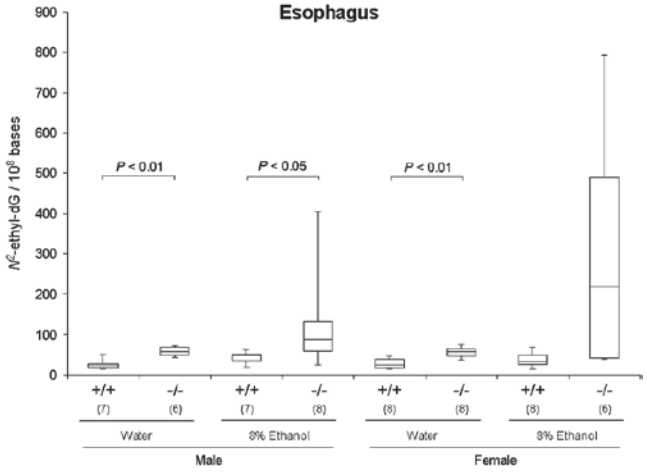

Figure 2. N^2-ethyl-dG levels in esophagus in *Aldh2*$^{+/+}$ and *Aldh2*$^{-/-}$ mice. Each number in parentheses indicates the number of samples.

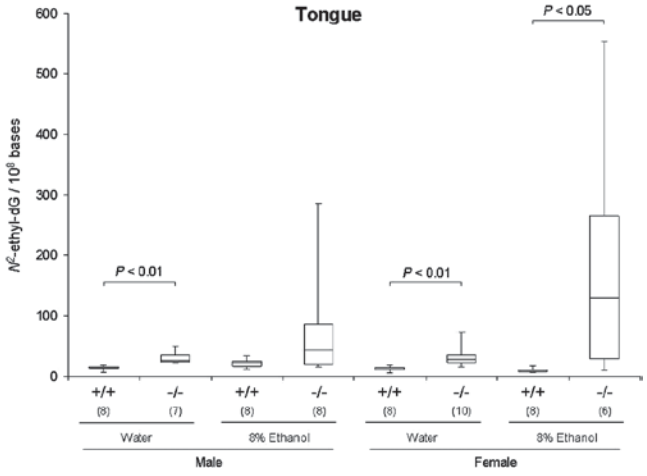

Figure 3. N^2-ethyl-dG levels in tongue in *Aldh2*$^{+/+}$ and *Aldh2*$^{-/-}$ mice. Each number in parentheses indicates the number of samples.

about 13 times higher in females than those of *Aldh2*$^{+/+}$ mice (Figure 3).

N^2-ethyl-dG levels in the submandibular gland

In the submandibular gland, N^2-ethyl-dG levels of water-treated male and female *Aldh2*$^{-/-}$ mice were relatively low compared with those of *Aldh2*$^{+/+}$ mice. Ethanol treatment raised the adduct levels about 7 fold in male and female *Aldh2*$^{-/-}$ mice. Also, the N^2-ethyl-dG levels of *Aldh2*$^{-/-}$ mice were higher than those of *Aldh2*$^{+/+}$ mice after ethanol treatment (2 times higher in males; 6 times higher in females) (Figure 4).

Increase in N^2-ethyl-dG levels in the three tissues

Figure 5 shows the net increase in N^2-ethyl-dG levels in all three tissues. Each value was calculated by subtracting the mean value for the water-treated group from that for the ethanol-treated group. Ethanol led to an increase in N^2-ethyl-dG levels in the three tissues of female

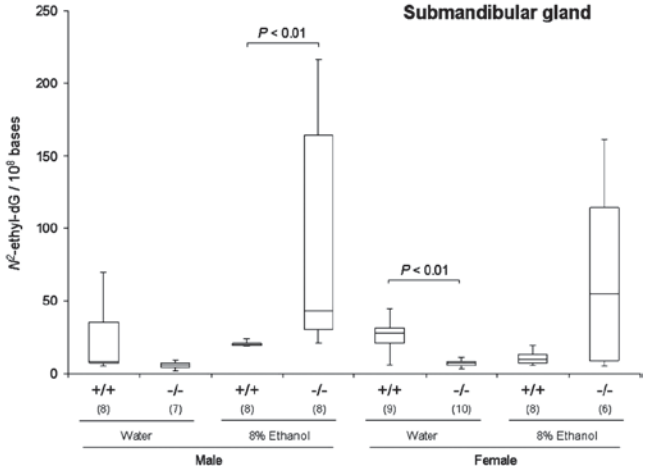

Figure 4. N^2-ethyl-dG levels in submandibular gland in *Aldh2*$^{+/+}$ and *Aldh2*$^{-/-}$ mice. Each number in parentheses indicates the number of samples.

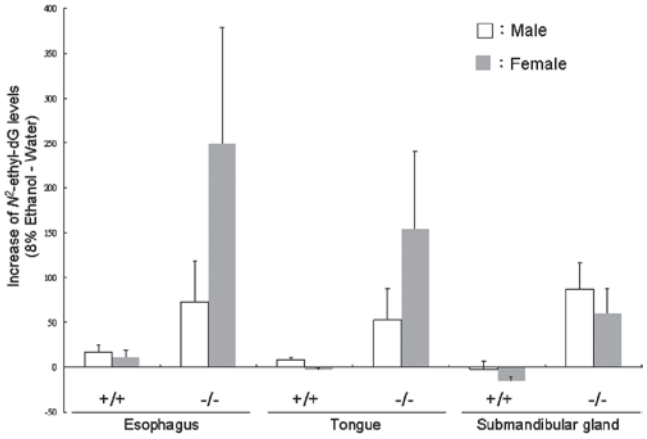

Figure 5. Increase in N^2-ethyl-dG levels in mice given 8% ethanol and water (8% Ethanol - Water). Each bar refers to the N^2-ethyl-dG level in mice given 8% ethanol minus that in mice given water. The error bars represent the standard error of the mean difference.

Aldh2$^{-/-}$ mice, in the order of esophagus, tongue and submandibular gland. In other words, the esophagus may be the site most susceptible to ethanol-induced carcinogenesis among the three tissues in female *Aldh2*$^{-/-}$ mice.

Discussion

In the present study, mice were treated with 8% ethanol for 14 months, and the body weights of the animals increased steadily during this period. No significant differences in body weight were observed between the mice given water mice and the ethanol-treated mice. Thus, it can be said that 8% ethanol does not have a severe effect on *Aldh2*$^{-/-}$ mice. In contrast, 40% ethanol treatment or 500 ppm of acetaldehyde inhalation caused remarkable weight loss in *Aldh2*$^{-/-}$ mice (Oyama et al. 2007a; Oyama et al. 2007b; Yu et al. 2009).

N^2-ethyl-dG was measured after the reduction of N^2-ethylidene-dG, a major acetaldehyde-derived DNA adduct, by $NaBH_3CN$. The analysis of N^2-ethyl-dG used

in the present study was highly sensitive with good precision (coefficient of variation = 8.5%) (Matsuda et al. 2006). The N^2-ethyl-dG was detected in all three tissues, even in water-treated mice. The reason why this adduct existed in ethanol-free mice might be that acetaldehyde was taken in from ambient air or food, or originated intrinsically from threonine metabolism (Ma et al. 1989). Our data demonstrated that basic levels of N^2-ethyl-dG were higher in *Aldh2*$^{-/-}$ mice than *Aldh2*$^{+/+}$ mice in both esophagus and tongue, but with a reverse result in submandibular gland. According to Matsuda et al. (2007), *Aldh2*$^{-/-}$ mice showed higher N^2-ethyl-dG levels in the liver, while *Aldh2*$^{+/+}$ mice showed higher levels in the stomach (Nagayoshi et al. 2009). Overall, these studies gave inconclusive results for baseline adduct levels in normal-drinking mice because of the low adduct levels.

The present study demonstrated that 8% ethanol treatment raised the level of N^2-ethyl-dG, which was produced from N^2-ethylidene-dG by reduction. The adduct levels were remarkably higher in *Aldh2*$^{-/-}$ than *Aldh2*$^{+/+}$ mice in all three tissues. Namely, N^2-ethylidene-dG adducts were generated by ethanol, dependent on the *Aldh2* genotype. This finding suggests greater potential DNA damage in *Aldh2*$^{-/-}$ mice than *Aldh2*$^{+/+}$ mice following ethanol treatment. The present animal study was consistent with human epidemiological studies.

Although the acetaldehyde associated with drinking alcohol strongly contributes to carcinogenesis in the oral cavity and esophagus, the exact mechanism involved remains unclear. One possible explanation is that salivary acetaldehyde was produced through the metabolism of ethanol, catalyzed by the ADH of oral microbes, mucosa, or salivary glands in the oral cavity (Purohit et al. 2005), and was mainly involved in alcohol-related carcinogenesis. As the acetaldehyde in saliva was in close contact with the mucosa of the oral cavity and able to enter the esophagus (Homann et al. 1997), these regions were continuously exposed to saliva with a high acetaldehyde concentration. After drinking 8% ethanol, *Aldh2*$^{-/-}$ mice might exhibited high acetaldehyde levels in saliva, which delivered acetaldehyde directly to the oral cavity and esophagus, resulting in acetaldehyde-derived DNA adduct formation.

Conclusions

The present study demonstrated that long-term consumption of ethanol leads to acetaldehyde-derived DNA adducts forming in the esophagus, tongue and submandibular gland in Aldh2-deficient mice. The results are consistent with epidemiological studies showing the risk of oral and esophageal cancer to be higher in ALDH2-deficient individuals who drink chronically.

Declaration of interest

This study was supported in part by Grants-in-aid from the Ministry of Education, Culture, Sport, Science and Technology of Japan (18390187 and 20590620 to T.O. and T.K.). It was also funded in partby a grant to research the association between the risk of upper aero-digestive tract cancer and alcohol-metabolizing enzymes, and its clinical significance (to T.O. and T.M.), number 20-10. The authors report no declarations of interest.

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