J Occup Health 2007; 49: 363–369

Journal of Occupational Health

Ethanol-Induced Oxidative DNA Damage and CYP2E1 Expression in Liver Tissue of *Aldh2* Knockout Mice

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Abstract: Ethanol-Induced Oxidative DNA Damage and CYP2E1 Expression in Liver Tissue of *Aldh2* Knockout Mice: Yong-Dae KIM, *et al.* Department of Preventive Medicine and Medical Research Institute, College of Medicine, Chungbuk National University, Republic of Korea—Excessive alcohol consumption is associated with increased risks of many diseases including cancer. We evaluated oxidative DNA damage in *Aldh2* +/+ and *Aldh2* –/– mice after they had been subjected to acute ethanol exposure. Olive tail moment, which was measured using a comet assay, was not increased by ethanol treatment in both *Aldh2* +/+ and *Aldh2* –/– mice. However, after controlling for the effect of ethanol exposure, the *Aldh2* genotype was a significant determinant for Olive tail moments. Although the ethanol treatment significantly increased the hepatic 8-OHdG generation in only *Aldh2* +/+ mice, the level of 8-OHdG was the highest in *Aldh2* –/– ethanol treated mice. The increase in the level of 8-OHdG was associated with hepatic expression of cytochrome P450 2E1 (CYP2E1). The levels of Olive tail moment and the hepatic 8-OHdG in the *Aldh2* –/– control group were significantly higher than those of the *Aldh2* +/+ control group. The level of CYP2E1 in liver tissue showed a similar pattern to those of the oxidative DNA damage markers. This study shows that acute ethanol consumption increases oxidative DNA damage and that expression of CYP2E1 protein may play a pivotal role in the induction of oxidative DNA damage. The finding that oxidative DNA damage was more intense in *Aldh2* –/– mice than in *Aldh2* +/+ mice suggests that ALDH2-deficient individuals may be more susceptible than wild-type ALDH2 individuals to ethanol-mediated liver disease, including cancer.

Received Feb 23, 2007; Accepted Jun 6, 2007
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(J Occup Health 2007; 49: 363–369)

Key words: Aldehyde dehydrogenase 2, Cytochrome P450 2E1, Oxidative stress, Olive tail moment, 8-Hydroxydeoxyguanosine

Excessive alcohol consumption is associated with increased risks of many diseases including cancer[1, 2]. There is evidence that the metabolism of ethanol gives rise to the generation of excess amounts of free radicals[3–5]. Acute ingestion of ethanol has been related to the formation of ROS[6, 7]. Although many pathways have been suggested to explain how ethanol induces oxidative stress, the exact mechanisms are still unknown.

Ingested ethanol is oxidized by cytosolic class I alcohol dehydrogenase (ADH) to acetaldehyde, which is subsequently oxidized by mitochondrial aldehyde dehydrogenase 2 (ALDH2) to produce acetate[8]. After ethanol consumption, the activity of the microsomal ethanol-oxidizing system (MEOS) is increased, with an associated increase in cytochrome P450 activity, especially that of cytochrome P450 2E1 (CYP2E1)[9, 10]. However, because the main enzyme for acetaldehyde metabolism is ALDH2[11], there is a possibility that the activity of the MEOS may differ according to the activity of ALDH2.

About 50% of Asians, including Koreans, lack ALDH2 activity because of a point mutation in the *ALDH2* gene[12, 13]. Individuals who regularly consume excessive quantities of alcohol have a greater risk of developing head and neck cancers such as esophageal, pharyngeal and oral cavity cancers if they are ALDH2 deficient than if they express wild-type ALDH2[14–16]. This suggests that oxidative stress and genetic damage caused by ethanol exposure may be more severe in ALDH2-deficient individuals than in those who express wild-type ALDH2, and that the level of CYP2E1 expression may be the cause of the difference between the genotypes in

ethanol-induced toxicity.

In this study, we used *Aldh2* knockout mice as a model of ALDH2-deficient humans to elucidate the effects of the *ALDH2* polymorphism on ethanol-induced oxidative stress. We evaluated the level of Olive tail moment, the concentration of 8-hydroxydeoxyguanosine (8-OHdG) in liver tissue and the expression of the CYP2E1 enzyme in liver microsomes in *Aldh2* +/+ and *Aldh2* –/– mice after they had been subjected to acute ethanol exposure by gavage.

Materials and Methods

Animals

Aldh2 –/– mice were generated as described previously[17]. Male mice with *Aldh2* +/+ and *Aldh2* –/– (C57BL/6J strains), 12-weeks-old, were used. The mice were housed individually in plastic mouse cages and had free access to standard rodent chow and water throughout the experiments. The colony room was maintained at 23~25°C under a 12 h light (07:00~19:00)–12 h dark (19:00~07:00) cycle. All procedures were performed according to the criteria outlined in the Guide for the Care and Use of Laboratory Animals prepared by the National Academy of Sciences and published by the National Institutes of Health.

ALDH2 genotyping and ethanol treatment

Genomic DNA extracted from the tip of the tail was tested for the presence of mutated and/or wild-type *Aldh2* alleles by PCR amplification as described previously[18]. Seven mice in each group received 40% ethanol (2 g/kg/d) in saline solution (0.5 m*l*/kg/d) by gavage for seven days. Control mice received saline only.

Comet analysis

The alkaline comet assay was used to measure DNA damage according to the method of a previous study[19] with slight modifications. Glass microscope slides with frosted ends were first coated with 1.0% (w/v) agarose and allowed to dry. Blood samples from mice were suspended in 0.5% low-melting-point agarose, and the resulting suspensions were applied to the slides using coverslips. The coverslips were removed after the agarose had hardened. Slides were then treated in the dark at 4°C with lysis buffer (1% Triton X-100, 10% DMSO, 89% stock lysing solution [2.8 M NaCl, 0.1 M Na_2EDTA, 0.01 M Trizma Base]) for 1 h and were subsequently rinsed in 0.4 M Tris, pH 7.5. Slides were then placed in the electrophoresis unit and covered with a fresh solution containing 300 mM NaOH and 1 mM EDTA, final pH >13.0, for 60 min. The slides were electrophoresed with a current of 300 mA for 20 min. Each slide was stained with ethidium bromide (2 μg/m*l*) for 5 min. Images were made of 50 cells per slide, and Olive tail moment [(Tail-mean–Head-mean)*Tail%DNA/100] was determined using Komet 4.0 Image Analysis and Data Capture software (Kinetic Imaging, Merseyside, England). An A549 lung epithelial cell line was used to compensate for variation between runs.

DNA isolation and measurement of 8-OHdG levels in liver tissue

The level of 8-OHdG in liver DNA extracted from *Aldh2* +/+ and *Aldh2* –/– mice after ethanol exposure for seven days was measured using an 8-OHdG ELISA kit (Jaica, Shizuoka, Japan). Briefly, DNA was extracted from liver tissue using the DNA Extractor WB kit (Wako, Osaka, Japan), digested with P1 nuclease (Sigma, St. Louis, MO), treated with alkaline phosphatase (Sigma) at 37°C for 1 h, and centrifuged. The ELISA was performed according to the manufacturer's instructions.

Immunoblotting

The relative level of CYP2E1 protein in liver tissue of the mice was measured using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting[9]. Liver microsome proteins were prepared by differential ultracentrifugation and stored at –70°C, before immunoblotting. Protein content was determined using the Bio-Rad Protein Assay Kit (Hercules, CA, USA) according to the manufacturer's instructions. Ten micrograms of liver microsomal proteins were separated using SDS-PAGE (10% polyacrylamide) and transferred onto Polyvinylidene fluoride (PVDF) membranes (Amersham, Buckinghamshire, UK) using a semi-dry system (Bio-Rad, Hercules, CA). Blots were immunolabeled with rabbit anti-human/rat CYP2E1 polyclonal antibody (Chemicon, Temecula, CA) which has been demonstrated to react with mice CYP2E1. Bound antibodies were detected by using horseradish peroxidase conjugated goat anti-rabbit IgG (Labfrontier, Suwon, Korea) and an enhanced chemiluminescence detection system (Amersham) according to the manufacturer's instructions. GAPDH was used as an internal standard to correct CYP2E1 band densities for differences in protein concentration. The densities of bands were quantified by using a photodensitometer (Roche Diagnostics, Basel, Switzerland).

Statistical analysis

Statistical analyses were performed using SPSS for Windows (Version 10.0). Differences between the means of two groups were tested by the Wilcoxon rank sum test. Pearson correlation coefficients were calculated among the level of the Olive tail moments, 8-OHdG in liver tissue, and CYP2E1 expression. Finally, we performed 2-way ANOVA test to evaluate the effects of the *Aldh2* genotype and ethanol exposure on the Olive

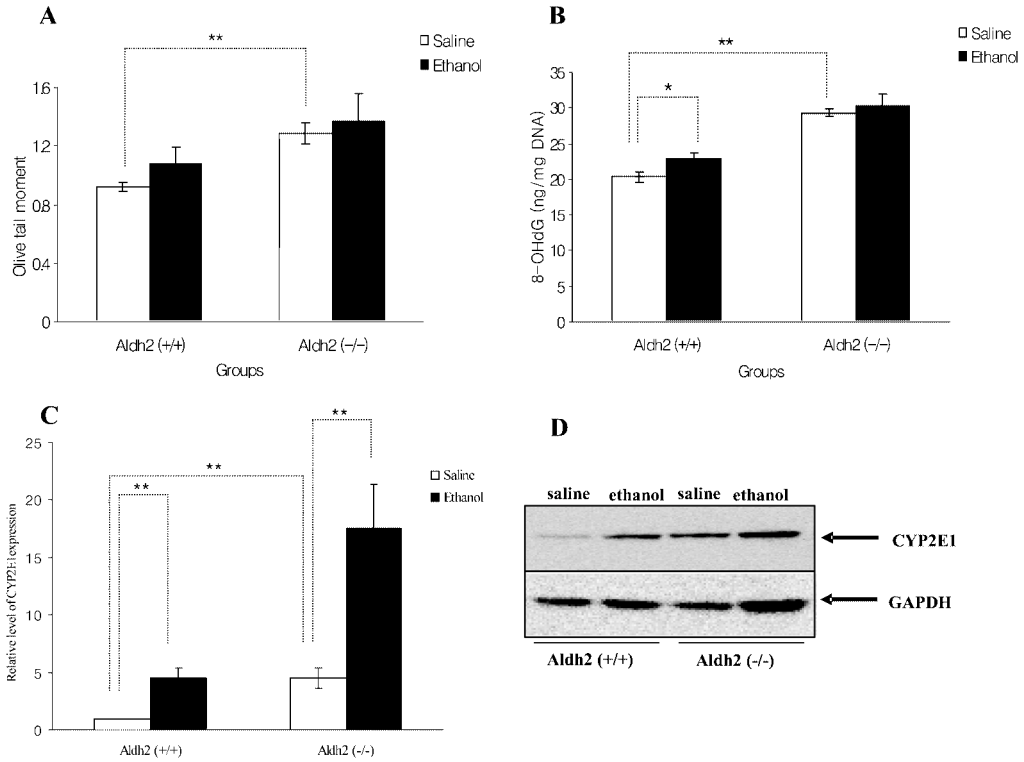

Fig. 1. Olive tail moment (mean ± S.D. of seven mice) of leukocytes (A), 8-hydroxydeoxyguanosine in liver tissue (B) and relative expressions of hepatic CYP2E1 (C and D) of mice treated with saline or 2 g/kg/d ethanol for 7 d. Protein (10 μg) samples from seven different livers for each treatment group were individually analyzed for CYP2E1 protein content by western blotting using an immunoreactive human/rat CYP2E1 polyclonal antibody (D). The mean CYP2E1 protein level of *Aldh2* +/+ control mice was assigned an index of 1 (C). Significant differences are indicated by **p*<0.05 and ***p*<0.01.

tail moments, the level of 8-OHdG in liver tissue, and CYP2E1 protein expression level.

Results

Figure 1A shows that Olive tail moment, an index of DNA damage, was not increased by ethanol treatment in both *Aldh2* +/+ and *Aldh2* –/– mice. However, the level of Olive tail moment was the highest in the ethanol treated *Aldh2* –/– group (1.37 ± 0.19). On the other hand, the level of Olive tail moment in the *Aldh2* –/– control group was significantly higher than that of the *Aldh2* +/+ control group (1.29 ± 0.07 vs. 0.92 ± 0.03, $p<0.05$). We evaluated 8-OHdG levels in liver tissue DNA isolated from *Aldh2* +/+ and *Aldh2* –/– mice after they had been subjected to acute ethanol treatment. The ethanol treatment significantly increased 8-OHdG generation in only *Aldh2* +/+ mice (20.29 ± 1.46 vs. 22.92 ± 1.66 ng/mg DNA, $p<0.05$) (Fig. 1B). However, the level of 8-OHdG was the highest in ethanol treated *Aldh2* –/– mice (30.25 ± 3.35 ng/mg DNA, Fig. 1B) and the level of 8-OHdG in *Aldh2* –/– control group was significantly higher than that of the *Aldh2* +/+ control group (29.34 ± 0.95 vs. 20.29 ± 1.46, $p<0.01$, Fig. 1B).

Figures 1C and D show the relative expression levels of CYP2E1 protein in *Aldh2* +/+ and *Aldh2* –/– mice treated with ethanol. The expression levels of hepatic CYP2E1 protein were respectively 4.53 and 3.88 times those of mice that were not exposed to ethanol ($p<0.01$). The *Aldh2* –/– control mice were shown to have higher CYP2E1 protein expression level by a factor of 4.50 compared with the *Aldh2* +/+ control group ($p<0.01$).

Figure 2 shows relationships between the Olive tail moments, 8-OHdG in liver tissue, and CYP2E1 expression. The level of 8-OHdG in liver tissue was significantly correlated with the expression of CYP2E1 protein (R=0.65, $p<0.001$; Fig. 2A). The Olive tail moments of leukocytes showed significant correlation with the level of 8-OHdG in liver tissue (R=0.54, $p<0.01$, Fig. 2B), and the Olive tail moments was also significantly correlated with the CYP2E1 expression (R=0.29, $p<0.05$, Fig. 2C).

We performed a 2-way ANOVA test to evaluate the

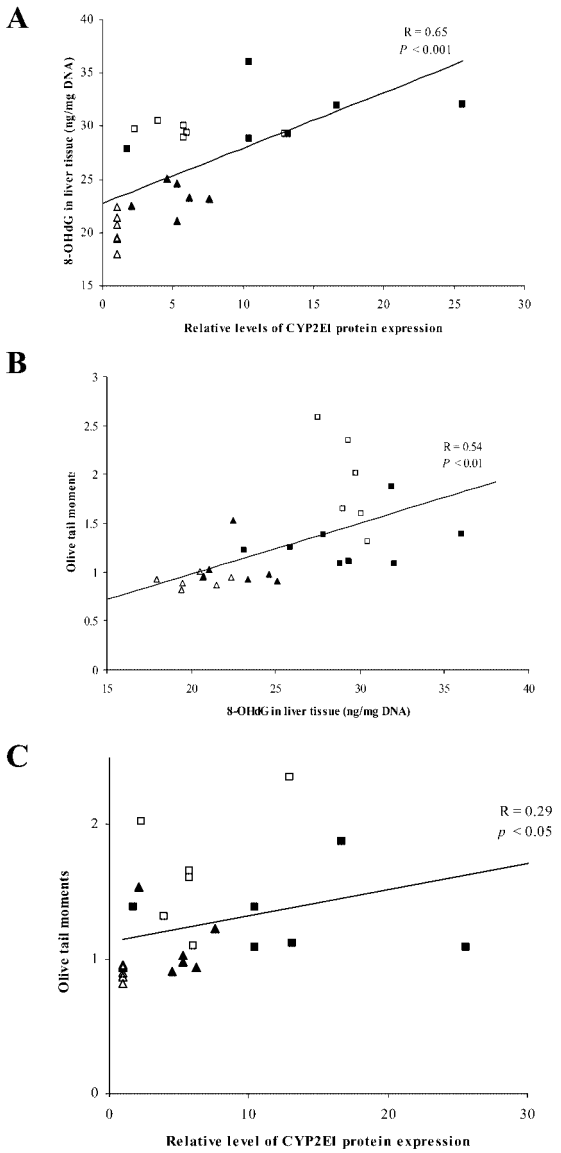

Fig. 2. The relationships between the relative expressions of CYP2E1 and level of 8-OHdG in liver tissue (A), the level of 8-OHdG in liver tissue and Olive tail moment (B) and the relative expressions of CYP2E1 and Olive tail moment in leukocyte (C). R=correlation coefficient. △; *Aldh2* +/+ control, ▲; *Aldh2* +/+ ethanol, □; *Aldh2* –/– control, ■; *Aldh2* –/– ethanol exposed group.

effects of the *Aldh2* genotype and ethanol exposure on the Olive tail moments, the level of 8-OHdG in liver tissue, and CYP2E1 protein expression level (Table 1). After controlling for the effect of ethanol exposure, the *Aldh2* genotype was shown to a significant determinant for Olive tail moments (p<0.001). On the other hand, the 8-OHdG level in liver tissue and CYP2E1 expression levels were affected not only by *Aldh2* genotype, but also by ethanol exposure.

Discussion

It is known that alcohol-induced liver damage is caused by oxidative stress and that ethanol-induced oxidative stress can be inhibited by antioxidants[20]. Zhang *et al*.[21] recently reported that a 24 h exposure to acetaldehyde significantly enhanced intracellular ROS generation in cultured myocytes. On the basis of these facts, we hypothesized that ALDH2-deficient individuals may be more susceptible to ethanol-induced oxidative stress than ALDH2 wild-type individuals because ALDH2-deficient individuals have higher blood acetaldehyde concentrations than ALDH2 wild-type individuals[11].

The comet assay is considered to be a rapid, sensitive and relatively simple method for detecting DNA damage at the level of individual cells[22]. Olive tail moment, which is a kind of main parameter of the comet assay and is defined as the product of the distance between the head and CG of DNA in the tail and the%DNA in the tail, is known to be the most informative feature of the comet image[23]. We used a comet assay to evaluate DNA damage in lymphocytes taken from *Aldh2* +/+ and *Aldh2* –/– mice after acute ethanol treatment. Olive tail moment was not increased by ethanol treatment in both *Aldh2* +/+ and *Aldh2* –/– mice. However, the level of Olive tail moment in the *Aldh2* –/– control group was significantly higher than that of the *Aldh2* +/+ control group. This may be a reason why the increase of Olive tail moment in *Aldh2* –/– mice by ethanol was not statistically significant in spite of the level of Olive tail moment being highest in the ethanol treated *Aldh2* –/– group.

8-OHdG, an oxidized nucleoside of DNA that induces GC-to-TC transversions, is a major product of oxidative DNA damage. Chronic ethanol treatment increases the level of 8-OHdG in rat liver DNA[24]. In this study, we

Table 1. Analysis of variance (2-way ANOVA) of the effects of the *Aldh2* genotype and ethanol exposure on the Olive tail moment, 8-OHdG in liver tissue, and CYP2E1 expression

Variables	Olive tail moment	8-OHdG in liver tissue	CYP2E1 expression
Aldh2 genotype	F=16.680 p<0.001	F=109.693 p<0.001	F=9.281 p<0.01
Ethanol exposure	F=1.444 N.S	F=5.111 p<0.05	F=4.275 p<0.05

evaluated 8-OHdG levels in liver tissue DNA isolated from *Aldh2* +/+ and *Aldh2* –/– mice after they had been subjected to acute ethanol treatment. Although the ethanol treatment significantly increased 8-OHdG generation in only *Aldh2* +/+ mice, the level of 8-OHdG was the highest in *Aldh2* –/– ethanol treated mice and the level of 8-OHdG in the *Aldh2* –/– control group was significantly higher than that of the *Aldh2* +/+ control group. In addition, there was a significant difference in the mean level of the 8-OHdG according to the *Aldh2* genotype and ethanol exposure (Table 1). These results suggest that *Aldh2* –/– mice may be more susceptible than *Aldh2* +/+ mice to ethanol induced oxidative DNA damage. The tail moment in alkaline single-cell gel electrophoresis reflects base modifications[25] as well as DNA strand breaks, because the oxidized purine (8-OHdG and others) and pyrimidine bases can be converted into additional DNA single-strand breaks[26]. Higher content of 8-OHdG in cells is expected to lead to a higher formation of DNA strand breaks, although the steady-state level of damage can be modulated by DNA repair[27]. In the present study, the Olive tail moments of leukocytes showed significant correlation with the level of 8-OHdG in liver tissue (Fig. 2B). This suggests the possibility that the generation of 8-OHdG by ethanol treatment in mice may accelerate the DNA single-strand breaks, although this hypothesis has to be proven by further investigations.

CYP2E1, a cytochrome P450 enzyme that is induced by ethanol and acetaldehyde, displays high NADPH oxidase activity and generates ROS[28]. The increase in ROS production and lipid peroxidation in microsomes from chronic ethanol-treated rats was blocked by chemical inhibitors of CYP2E1 and by anti-CYP2E1 immunoglobulin G[2, 28]. CYP2E1 can generate ROS even in the absence of substrate[29]. Therefore, we evaluated CYP2E1 protein expression levels in liver tissues of mice. Expression levels of CYP2E1 protein in *Aldh2* +/+ and *Aldh2* –/– mice treated with ethanol were respectively 4.53 and 3.88 times greater that those of the mice that were not exposed to ethanol (Fig. 1C).

The expression of CYP2E1 protein correlated with the level of 8-OHdG in liver tissue and Olive tail moments (Fig. 2A, C). In addition, the *Aldh2* genotype and the ethanol exposure significantly affected the CYP2E1 expression level in liver tissue (Table 1). These results suggest that the CYP2E1 proteins play pivotal roles in the induction of ethanol-induced oxidative DNA damage.

In this study, we measured Olive tail moment in leukocytes, and 8-OHdG and CYP2E1 in hepatocytes. This difference in the cell type on which comet assay, and measurements of 8-OHdG and CYP2E1 expression level were preformed might affect the associations between those markers. However, considering the fact that ingested ethanol and their metabolites can circulate in blood and damage leukocytes as well as hepatocytes, it is not meaningless to evaluate relationships between these markers even though they were measured in different cell types.

It is interesting that the *Aldh2* –/– mice had greater oxidative DNA damage than the *Aldh2* +/+ mice when not exposed to ethanol treatment. Olive tail moment of the *Aldh2* –/– control group was significantly higher than that of the *Aldh2* +/+ control group ($p<0.01$, Fig. 1A). The levels of 8-OHdG in liver tissue of the *Aldh2* –/– control group were also significantly higher than those of the *Aldh2* +/+ control group ($p<0.01$, Fig. 1B). Furthermore, CYP2E1 protein expression in liver tissue of the *Aldh2* –/– control group was significantly higher than that of the *Aldh2* +/+ control group ($p<0.01$, Fig. 1C). These results are consistent with those of our preliminary study which showed that *Aldh2* –/– mice have higher CYP2E1 and 2B1/2 protein expression levels than *Aldh2* +/+ mice in the absence of any chemical treatment (4.19 and 2.89 times, respectively)[9]. It is known that ethanol is produced in mammalian tissues endogenously[30], and that CYP2E1 metabolizes a wide variety of endogenous substrates other than ethanol[31]. Therefore, there is a possibility that greater expression of CYP2E1 enzyme was induced by endogenous ethanol in *Aldh2* –/– mice to compensate for the absence of the ALDH2 enzyme.

In this study, ethanol exposure tended to increase the levels of Olive tail moment and 8-OHdG in both *Aldh2* +/+ and *Aldh2* –/– mice compared to their respective controls. *Aldh2* –/– mice, nevertheless, showed lack of significance in the increase of Olive tail moment and 8-OHdG, because the level of those markers in the *Aldh2* –/– control group were significantly higher than those of the *Aldh2* +/+ control group. Therefore, we adopted a 2-way ANOVA analysis in order to evaluate the effect of ethanol exposure on the level of those markers after controlling for the ALDH2 genotype. The result showed that 8-OHdG level was significantly affected by ethanol exposure. This fact suggests that acute ethanol consumption increases oxidative DNA stress. However, we cannot exclude the possibility that various antioxidant systems, such as SOD, catalase and glutathione peroxidase, may also affect the induction of oxidative stress by ethanol exposure.

In conclusion, this study has shown that acute ethanol consumption increases oxidative DNA damage and that expression of the CYP2E1 protein plays a pivotal role in the induction of oxidative DNA damage. That oxidative DNA damage was more intense in *Aldh2* –/– mice than in *Aldh2* +/+ mice suggests that ALDH2-deficient individuals may be more susceptible than wild-type ALDH2 individuals to ethanol-mediated liver diseases, including cancer.

Acknowledgments: This study was supported by Korea-

Japan Joint Research Project under the KOSEF-JSPS Cooperative Program (F01-2003-000-20031-0).

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