Association between Aldehyde Dehydrogenase-2 Gene Polymorphisms and Consumption of Alcohol in the Precursor Lesions of Esophageal Squamous Cell Carcinoma

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Abstract: The genetic polymorphisms of aldehyde dehydrogenase-2 (ALDH2) affect the metabolism of alcohol. The inactive $ALDH2^{*1/2*2}$ heterozygote genotype increases the risk of esophageal squamous cell carcinoma because of accumulation of acetaldehyde. The aim of this prospective study was to investigate whether daily alcohol consumption and the genetic polymorphisms of ALDH2 were risk factors for esophageal squamous intraepithelial neoplasia, as low-grade intraepithelial neoplasia (LGIN) is considered to be an initial lesion of esophageal squamous cell carcinoma. Lugol chromoendoscopy was performed in 400 subjects at Showa University Hospital from January 2004 to August 2008. The effects of smoking, alcohol consumption, and ALDH2 genotype were evaluated in subjects with high-grade intraepithelial neoplasia (HGIN), LGIN, and controls without neoplasia. Of the 400 subjects, 32 (8%) had HGIN, 21 (5%) had LGIN, and 347 (87%) were controls. There were no significant differences in the proportion of male subjects, or the prevalence of smoking, drinking, or the $ALDH2^{*1/2*2}$ genotype between LGIN subjects and controls. In contrast, the prevalence of $ALDH2^{*1/2*2}$ was significantly higher in HGIN (24/32, 75%) than in LGIN subjects (8/21, 38%; $P = 0.007$). Furthermore, the prevalence of alcohol consumption plus $ALDH2^{*1/2*2}$ differed significantly between HGIN (20/25, 80%) and LGIN (3/12, 25%; $P = 0.001$), while there was no significant difference between LGIN and controls. Daily alcohol consumption and the $ALDH2^{*1/2*2}$ genotype were not risk factors for LGIN, however, alcohol consumption was a high-risk factor for HGIN in subjects with $ALDH2^{*1/2*2}$.

Key words: ALDH2, esophagus, precancerous lesions, risk factor

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Introduction

The International Agency for Research on Cancer has reported that consumption of alcoholic beverages is related to the development of squamous cell carcinoma in the oral cavity, pharynx, and esophagus\(^1\). In addition to the established risk factors of male gender, alcohol drinking, smoking, and lower intake of vegetables and fruit, there is growing evidence of a link between the risk of esophageal squamous cell carcinoma (ESCC) and the presence of the inactive heterozygous aldehyde dehydrogenase-2 (ALDH2) genotype encoded by \(ALDH2^*1/2^*2\). ALDH2 is the key enzyme in the elimination of acetaldehyde, which is an established animal carcinogen\(^6,7\), and is produced after drinking alcohol\(^8\). In persons with \(ALDH2^*1/2^*2\), a mutant allele seen in East Asian populations\(^8\), the ALDH2 enzyme is inactive, and since the body fails to metabolize acetaldehyde rapidly, excessive amounts of acetaldehyde accumulate\(^9\).

Malignant transformation of human esophageal epithelium is a multi-step progressive process\(^10\)–\(^20\). Various precancerous lesions, including both dysplastic (i.e. low-grade or high-grade intraepithelial neoplasia, and cancer in situ) and non-dysplastic lesions (i.e. basal cell hyperplasia, chronic esophagitis, and Lugol-unstained lesions with non-dysplastic epithelium (LULs-NDE)) have been proposed. The relationship between these lesions and \(p53\) gene mutations or \(p53\) overexpression has been investigated\(^10\)–\(^18\). The previously reported frequency of \(p53\) mutations in human ESCC is approximately 50%\(^17,18,21\). Similar frequencies of \(p53\) mutations have been reported in LGIN (36%–67%), suggesting that LGIN has already acquired malignant potential\(^12,17,18\). From the results of a 13-year follow-up study, a significant number of both LGIN and HGIN were confirmed to transform into ESCC rather than basal cell hyperplasia or chronic esophagitis\(^22\). In Wang’s study, squamous dysplasia and carcinoma in situ were the only histological lesions associated with a significantly increased risk of developing ESCC\(^22\). Increasing grades of dysplasia were strongly associated with increasing risk, indicating that the histological grading was clinically meaningful\(^22\). Therefore, LGIN has been established as an initial ESCC lesion worldwide.

In contrast to ESCC however, the causal factors and risk factors of esophageal intraepithelial neoplasia are unclear. The aim of this prospective study was to investigate whether daily alcohol consumption and \(ALDH2^*1/2^*2\) genotype were risk factors for esophageal squamous intraepithelial neoplasia.

Patients and Methods

Patients

All subjects were prospectively enrolled on the basis of the following eligibility criteria between January 2004 and August 2008. Adults, aged between 30–85 years, who received screening endoscopy for a health checkup, with a performance status of ‘zero’ according to the Eastern Cooperative Oncology Group, and who had no symptoms of dysphagia, abdomi-
nal pain, chest and/or back pain, or vomiting were eligible for the study. Subjects who had active malignant disease, and who had undergone esophagectomy or chemoradiotherapy for ESCC and gastrectomy were excluded. After endoscopic observation, subjects who had esophageal varices, Barrett’s esophagus, or reflux esophagitis were also excluded. This study was approved by the institutional review board of Showa University Hospital. All subjects provided written informed consent before enrollment.

Drinking and smoking habits

All subjects were interviewed using a structured questionnaire, which aimed to obtain detailed drinking and smoking habits, such as the average amount of pure ethanol consumed (g/day), duration of drinking (years), total amount of ethanol consumed (kg), average number of cigarettes smoked per day, duration of smoking (years), and smoking index. Taking into account the different ethanol concentrations, alcohol consumption was estimated as the average amount of ingested alcohol for every drinking day in grams of pure ethanol per day. The smoking index was calculated as the average number of cigarettes smoked per day multiplied by the total number of smoking years. The interviews took place immediately after endoscopic examination at the Endoscopy Center. Drinkers and smokers were defined as subjects who drank more than 5 days or more than 100 g of pure ethanol a week and smoked every day or more than 50 cigarettes a week, respectively. Drinkers and smokers who had ceased drinking and smoking for more than 5 years before enrollment were categorized as ex-drinkers and ex-smokers, respectively. Non-drinkers and non-smokers were defined as subjects who never used or who only rarely used alcohol and cigarettes, respectively.

Endoscopic examination

Videoendoscopy (Q240 or XQ240, Olympus, Tokyo, Japan) following Lugol solution spraying was performed on all subjects. After ordinary endoscopic observation, 5–10 ml of 2.0% glycerin-free Lugol’s iodine solution was sprayed from the gastroesophageal junction to the upper esophagus using a plastic spray catheter passed through the biopsy channel of the endoscope. The whole esophagus was then observed again and epithelial areas were categorized as unstained, normally stained, or over-stained. We defined LULs as areas either staining less intensely than normally-stained epithelium or completely unstained; this group of lesions included superficial carcinoma, intraepithelial neoplasia, and esophagitis. Samples were taken from all LULs and normal Lugol-staining background epithelium to confirm endoscopic findings.

Histological analysis

The biopsied tissues, stained with hematoxylin and eosin (H & E), were read by one pathologist (M.K.), without knowledge of the subject’s history or visual endoscopic findings.
Histological criteria for normal epithelium, esophagitis, basal cell hyperplasia, LGIN, HGIN, and squamous cell carcinoma were based on previous descriptions. All LULs were classified into squamous cell carcinoma, intraepithelial neoplasia, or LULs-NDE. LULs-NDE was defined as Lugol unstained lesions, without neoplastic components. Dysplasia was characterized by nuclear atypia (enlargement, pleomorphism, and hyperchromasia), and loss of normal cell polarity. The grade of intraepithelial neoplasia was defined as follows: mild, abnormal cell maturation in the lower one-third; moderate, abnormal cell maturation in the lower two-thirds; or severe, abnormal cell maturation in the whole epithelium from the basal cell layer. LGIN was defined as mild to moderate intraepithelial neoplasia, and HGIN was defined as severe intraepithelial neoplasia and carcinoma.

**DNA extraction**

Normal Lugol-staining background epithelium from the esophagus was collected by endoscopic examination. Samples were stored at −80°C until analysis. Genomic DNA was extracted from the biopsy specimens by conventional phenol-chloroform examination. The extracted genomic DNA was stored at 4°C until analysis for the genetic polymorphisms of ALDH2.

**ALDH2 genotyping**

Genotyping of ALDH2 polymorphism (Glu487Lys variant, rs671) was screened using the TaqMan fluorogenic 5’ nuclease assay (Applied Biosystems, Foster City, CA, USA). The final volume of each polymerase chain reaction (PCR) was 25 μl, containing 2 ng of genomic DNA and 12.5 μl TaqMan Universal PCR Master Mix, with 1.25 μl of 20 × TaqMan Drug Metabolism Genotyping Assay Mix (Assay ID C_11703892_10). Terminal cycle conditions were as follows: 50°C for 2 min to activate the uracil N-glycosylase and to prevent carry-over contamination, 95°C for 10 min to activate the DNA polymerase, followed by 50 cycles of 92°C for 15 sec and 60°C for 90 sec. All PCR amplifications and endpoint fluorescent readings were performed on an ABI PRISM 7900 HT Sequence Detection System (Applied Biosystems). Duplicate samples and negative controls were included to ensure accuracy of genotyping. The genotypes corresponding to homozygous for the ALDH2 *1 and ALDH2 *2 alleles are described here as ALDH2 *1/2 *1 and ALDH2 *2/2 *2, respectively, and the heterozygous genotype as ALDH2 *1/2 *2.

**Statistical analysis**

All subjects were classified into three groups (HGIN, LGIN, or controls without dysplasia). Subjects with LULs-NDE were classified as controls. Data for age, and details of alcohol consumption and smoking were expressed as mean ± standard deviation. For categorical variables such as alcohol consumption and smoking, quantitative data were compared between HGIN and LGIN or LGIN and controls using Mann-Whitney tests Demographic
data (drinking, smoking status, and ALDH2 polymorphism) were compared using chi-square tests between HGIN and LGIN or LGIN and controls. Ex-smokers and ex-drinkers were analyzed statistically as smokers and drinkers. A $P$ value of $<0.05$ was considered statistically significant.

**Results**

**Characteristics of subjects**

Between January 2004 and August 2008, 400 subjects were enrolled in this prospective study. Of the 400 subjects, there were 244 males and 156 females, with an average age of 66 years, ranging from 35–85 years. Among the 400 subjects, 32 (8%) were classified with HGIN, 21 (5%) with LGIN, and 347 (87%) were classified as controls without neoplastic lesions. Thirty (94%) of the male subjects were classified as HGIN, 12 (57%) as LGIN, and 202 (58%) as controls. Alcohol consumption occurred in 78 (25/32) of HGIN subjects, 57 (12/21) of LGIN subjects, and 50 (172/347) of controls. Cigarette smoking occurred in 91 (29/32) of HGIN subjects, 62 (13/21) of LGIN subjects, and 53 (183/347) of controls. The $ALDH2^{1/2\ast2}$ heterozygote genotype was present in 75 (24/32) of subjects in the HGIN group, 38 (8/21) of the LGIN group, and 34 (118/347) of the control group.

**Comparison between HGIN and LGIN**

The proportion of male subjects was significantly higher in the HGIN group than the LGIN group ($P=0.001$, Table 2). The prevalence of smoking was also significantly higher in HGIN subjects than in LGIN subjects ($P=0.01$). Seventy-eight percent of HGIN subjects and 57% of LGIN subjects consumed alcohol, but there was no significant difference between the two groups ($P=0.10$). In contrast, the prevalence of $ALDH2^{1/2\ast2}$ was significantly higher in HGIN subjects than in LGIN subjects ($P=0.01$). Although the $ALDH2^{1/2\ast2}$ heterozygote genotype was found in 20 out of 25 (75%) HGIN subjects with alcohol consumption, only 3 out of 12 (25%) subjects consuming alcohol from the LGIN group had the heterozygous genotype. Thus, the prevalence of alcohol consumption plus $ALDH2^{1/2\ast2}$ differed significantly between HGIN and LGIN subjects ($P=0.001$, Table 2). Furthermore, there were significant differences in the amount of ethanol per day ($P=0.01$), total amount of ethanol ($P=0.004$), duration of smoking ($P<0.001$), and smoking index ($P=0.02$) between subjects in the HGIN and LGIN groups. However, there were no significant differences in duration of drinking, and the number of cigarettes smoked per day between HGIN and LGIN groups.

**Comparison between LGIN and controls**

In contrast, there were no significant differences in the proportion of male subjects, the prevalence of smoking or drinking, or the prevalence of $ALDH2^{1/2\ast2}$ between LGIN...
Table 1. Characteristics of HGIN, LGIN, and control groups

<table>
<thead>
<tr>
<th></th>
<th>HGIN</th>
<th>LGIN</th>
<th>Control</th>
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<tbody>
<tr>
<td>Number of subjects</td>
<td>32</td>
<td>21</td>
<td>347</td>
</tr>
<tr>
<td>Gender (male/female)</td>
<td>30/2</td>
<td>12/9</td>
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<tr>
<td>Mean age (y)</td>
<td>69.7 ± 8.7</td>
<td>67.6 ± 7.1</td>
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<td>53–80</td>
<td>35–85</td>
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<td>Drinking (n)</td>
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<tr>
<td>Not drinking (n)</td>
<td>7</td>
<td>9</td>
<td>175</td>
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<tr>
<td>Smoking (n)</td>
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<td>13</td>
<td>183</td>
</tr>
<tr>
<td>Not smoking (n)</td>
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<td>164</td>
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<tr>
<td>Amount of ethanol (g/day)</td>
<td>72.6 ± 80.6</td>
<td>25.7 ± 34.2</td>
<td>21.5 ± 30.2</td>
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<tr>
<td>Duration of drinking (y)</td>
<td>32.9 ± 19.2</td>
<td>25.1 ± 22.6</td>
<td>21.6 ± 21.7</td>
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<td>Total amount of ethanol (kg)</td>
<td>1049 ± 1073</td>
<td>345 ± 452</td>
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<td>Number of cigarettes (per day)</td>
<td>19.9 ± 12.8</td>
<td>14.1 ± 13.2</td>
<td>12.2 ± 14.5</td>
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<tr>
<td>Duration of smoking (y)</td>
<td>40.5 ± 15.9</td>
<td>22.6 ± 19.5</td>
<td>179 ± 19.4</td>
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<td>Smoking index</td>
<td>912 ± 753</td>
<td>505 ± 483</td>
<td>407 ± 532</td>
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</table>

ALDH2 genotype

| ALDH2*1/2*1 | 6 | 11 |
| ALDH2*1/2*2 | 24 | 8 |
| ALDH2*2/2*2 | 2 | 2 |

Abbreviations: ALDH2, aldehyde dehydrogenase-2; HGIN, high-grade intraepithelial neoplasia; LGIN, low-grade intraepithelial neoplasia

Table 2. Comparison between HGIN and LGIN groups

<table>
<thead>
<tr>
<th></th>
<th>HGIN</th>
<th>LGIN</th>
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<td>Number of subjects</td>
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</tr>
<tr>
<td>Gender</td>
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<td>9</td>
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<tr>
<td>Alcohol</td>
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<tr>
<td></td>
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<td>9</td>
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<td>Cigarette</td>
<td>Smoking</td>
<td>29</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td>Not smoking</td>
<td>3</td>
<td>8</td>
</tr>
</tbody>
</table>

ALDH2 genotype

| ALDH2*1/2*1 | 6  | 11 |
| ALDH2*1/2*2 | 24 | 8  | 0.007 |
| ALDH2*2/2*2 | 2  | 2  |

ALDH2 genotype of subjects consuming alcohol

| ALDH2*1/2*1 | 5  | 9  |
| ALDH2*1/2*2 | 20 | 3  | 0.001 |
| ALDH2*2/2*2 | 0  | 0  |

Abbreviations: ALDH2, aldehyde dehydrogenase-2; HGIN, high-grade intraepithelial neoplasia; LGIN, low-grade intraepithelial neoplasia
subjects and controls (Table 3). The prevalence of alcohol consumption plus ALDH2*1/2*2 did not differ significantly between LGIN subjects and controls (Table 3). Furthermore, there were no significant differences in the amount of ethanol per day, duration of drinking, total amount of ethanol, number of cigarettes smoked per day, duration of smoking, or the smoking index between LGIN subjects and controls.

**Discussion**

We investigated whether the ALDH2*1/2*2 heterozygote genotype is a risk factor for intraepithelial neoplasia, which is considered to be an initial lesion of ESCC. The prevalence of ALDH2*1/2*2, alcohol consumption and cigarette smoking did not differ significantly between LGIN and controls. No significant differences were also seen in the amount or duration of alcohol consumption and cigarette smoking between LGIN and controls. Furthermore, the prevalence of alcohol consumption plus the ALDH2*1/2*2 genotype was not significantly different between LGIN subjects and controls. Therefore, we propose that alcohol consumption and cigarette smoking, and also the ALDH2*1/2*2 heterozygote genotype are not risk factors for LGIN.

In contrast, the prevalence of ALDH2*1/2*2 was significantly higher among HGIN than LGIN subjects. In this study, ALDH2*1/2*2 was found in 75% (24/32) of HGIN subjects, which is similar to the 51.3%–72.5% of ESCC patients worldwide reported to have ALDH2*1/2*2. Although no significant difference was seen in alcohol consumption between HGIN and LGIN subjects, the prevalence of ALDH2*1/2*2 in the subjects that consumed
alcohol differed significantly between the two groups. We suggest that daily alcohol consumption is a risk factor for HGIN in subjects with the $ALDH2^{*1/2*2}$ heterozygote genotype.

The effects of cigarette smoking, alcohol consumption, and the ALDH2 genotype on the occurrence of LGIN are quite different to HGIN, although LGIN is able to transform into HGIN via a multi-step progressive process $^{10-20}$. In this study, significant differences were seen in the amount of ethanol consumed per day, and the total ethanol intake between LGIN and HGIN subjects, while there were no significant differences in the amount or duration of alcohol consumption between LGIN subjects and controls. From our results, we suggest that long-term exposure to a large amount of alcohol will lead to the occurrence of HGIN in subjects with the $ALDH2^{*1/2*2}$ genotype. In contrast, we believe that LGIN can occur as a result of various factors, since alcohol consumption and cigarette smoking were not significantly related to the occurrence of LGIN. Furthermore, results from a 13-year follow-up study revealed that patients with mild dysplasia and severe dysplasia had relative risks of 2.9 and 28.3 for ESCC, respectively $^{22}$. Among those with mild dysplasia, 76% did not progress to ESCC despite the long duration of follow-up. Thus, the risk of ESCC in subjects with LGIN is very low and we suggest that most cases of LGIN will not progress to HGIN or ESCC. Therefore we propose that there is a difference between the causal mechanisms of LGIN and HGIN.

In summary, we completed a prospective study in which we collected questionnaire data, chromoendoscopic findings, and biological samples, and in which all subjects received the gold standard examination (endoscopy with Lugol-staining and biopsy) for esophageal squamous intraepithelial neoplasia. We propose that there are differences between the risk factors for LGIN and HGIN. Documenting the risk factors for the precursor lesions of ESCC should assist in the development of effective prevention, early detection, and treatment strategies for this disease.

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