Association of *aldehyde dehydrogenase 2* gene polymorphism with multiple oesophageal dysplasia in head and neck cancer patients

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Abstract

Background—Multiple occurrences of oesophageal dysplasia are frequently observed in head and neck cancer patients, and closely associated with alcohol consumption. Acetaldehyde, the first metabolite of ethanol, is thought to play an important role in the carcinogenesis of the upper aerodigestive tract.

Aim—To investigate if genetic polymorphism in alcohol metabolising enzymes (ADH3, alcohol dehydrogenase 3; ALDH2, aldehyde dehydrogenase 2) is associated with oesophageal multiple dysplasia in head and neck cancer patients.

Methods—Thirty one consecutive patients with head and neck cancer were included in the study. Multiple oesophageal dysplasia was detected endoscopically as multiple Lugol voiding lesions (multiple LVL) using the Lugol dye staining method. The *ADH3* and *ALDH2* genotypes were determined by polymerase chain reaction-restriction fragment length polymorphism.

Results—Among the 31 patients with head and neck cancer, 17 had multiple LVL. Multiple LVL were closely associated with a second primary oesophageal carcinoma in head and neck cancer patients (odds ratio 60.7, 95% CI 5.6–659). Furthermore, the mutant *ALDH2* allele was significantly more prevalent in patients with multiple LVL (65% *v* 29%; p<0.05) whereas no difference was observed in *ADH3* polymorphism.

Conclusions—The mutant *ALDH2* allele appears to be a risk indicator for multiple LVL in head and neck cancer patients. Accumulation of acetaldehyde due to low ALDH2 activity may play a critical role in cancerous changes throughout the mucosa in the upper aerodigestive tract.

(*Gut* 2000;47:256–261)

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Accepted for publication
8 February 2000

Squamous epithelial cell dysplasia has a high potential to progress to malignancy. Premalignant lesions of squamous cell carcinoma in the upper aerodigestive tract are similar to those in the neck of the uterus and skin.[1–3] Dysplastic lesions and squamous cell carcinoma of the oesophagus can be easily detected endoscopically by spraying Lugol dye solution[4]: the dye reacts with glycogen in the cells of the normal epithelium and leaves dysplastic and cancerous lesions unstained.

In patients with head and neck cancer, we often encountered an unusual endoscopic finding in that a larger number of Lugol unstained lesions spread throughout the oesophageal mucosa. This endoscopic finding was rare in other patients, and we discriminated these multiple Lugol voiding lesions (multiple LVL) from a common single Lugol unstained lesion. As similar lesions surrounding the surgically resected oesophageal carcinoma were frequently observed in patients with a second primary head and neck cancer,[5] such lesions were thought to be related to the development of squamous cell carcinoma in the oesophagus. Therefore, we thought it would be useful to determine the aetiology of multiple LVL in head and neck cancer patients.

Regarding multiple occurrences of neoplastic changes in the upper aerodigestive tract, genetic and/or environmental factors are considered to play an important role in the pathogenesis. However, to date, genetic abnormalities (such as replication errors and *p53* mutation) have not been associated with this lesion.[6–8] Alcohol consumption and cigarette smoking are generally accepted as major environmental risk factors for single and multiple occurrences of head and neck cancer and oesophageal cancer.[5 9–11] Acetaldehyde, the first oxidative metabolite of ethanol, is recognised as a carcinogen and has a tumour promoting effect on the upper aerodigestive tract in experimental animal models[12 13] although the precise mechanism is unknown. The in vivo concentration of acetaldehyde depends on the activities of two ethanol metabolising enzymes, alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH), which oxidise ethanol to acetaldehyde and acetaldehyde to acetate, respectively.[14]

It was recently reported that individuals homozygous for the genotype $ADH3^{1-1}$ have an increased risk of alcohol related oral and pharyngeal cancers.[15] In Japanese alcoholics, the mutant *ALDH2* allele, which is found only in Orientals at a frequency of approximately 50% and associates with intolerance to alcohol,
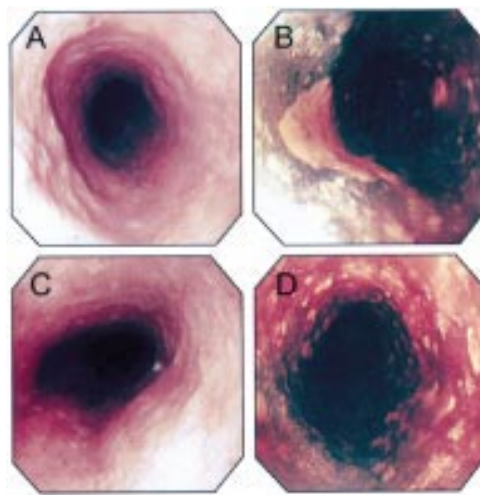
Figure 1 Endoscopic images of the oesophagus in a patient with head and neck cancer (A–D). Conventional endoscopic images (A, C) showed no abnormalities. After application of Lugol dye solution, multiple well defined, irregular shaped, yellowish white multiple Lugol voiding lesions (LVL) were seen scattered throughout the oesophageal mucosa (B, D).

was also reported to be a risk factor for multiple cancers of the upper aerodigestive tract.[16–18]

In this study, we assessed the association of multiple LVL with the occurrence of a second primary oesophageal cancer and genetic polymorphism of the *ADH3* and *ALDH2* genes, in patients with head and neck cancer.

Methods

PATIENTS

Thirty one consecutive patients (27 men, four women; mean age 63 years (SD 10)) with head and neck cancer who underwent oesophagoscopy at the Department of Gastrointestinal Oncology and Gastroenterology, National Cancer Center Hospital East, Japan, were screened using the endoscopic Lugol dye staining method and the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method. In this study, the anatomical sites of head and neck cancer included only the oropharyngolaryngeal region: 15 had pharyngeal or larynx cancer, and the remaining 16 had cancer of the oral cavity. All head and neck cancers were histologically confirmed to be squamous cell carcinomas. We used the same questionnaire to define drinking and smoking habits, and family history of cancer for all patients. Patients who abstained from drinking any alcoholic beverage and from cigarette smoking throughout their life were classified as non-drinkers and non-smokers. Daily alcohol consumption was calculated as grams per day. All biopsy samples were obtained with informed consent and in accordance with the Helsinki Declaration.

ENDOSCOPIC SCREENING METHODS AND DEFINITION OF MULTIPLE LVL

Endoscopic screening of multiple LVL in the oesophagus was performed using the Lugol dye staining method, as described previously,[19] using an electronic endoscope (Q230, Olympus Optical Co. Ltd, Tokyo, Japan). When a large number of well defined irregular shaped Lugol unstained lesions were observed endoscopically throughout the entire oesophageal mucosa after application of the Lugol dye solution, the patient was defined as having multiple LVL (fig 1).

TISSUE SAMPLE COLLECTION

Normal oesophageal tissues that stained positively with Lugol dye solution were collected endoscopically. Samples were stored at −80°C until analysis. Genomic DNA was prepared from biopsy specimens by conventional phenol-chloroform extraction. The amount of DNA was determined spectrophotometrically.

PCR-RFLP ANALYSIS OF *ADH3* GENE POLYMORPHISM

The *ADH3* genotype was determined using PCR-RFLP.[20] Genomic DNA was initially digested with *Nla*III (TOYOBO, Osaka, Japan) to prevent amplification of closely related *ADH1* and *ADH2* genes. PCR was performed in a 50 μl volume of PCR mixture containing 100 ng of DNA, 10 mM Tris HCl (pH 8.3), 50 mM KCl, 3 mM $MgCl_2$, 50 pmol of each primer (sense: 5'-GCTTTAAGAGTAAATATTCTGTCCC-3'; antisense: 5'-AATCTACCTCTTTCCGAAGC-3'), 250 μM of each dNTP, and 5 U of *Taq* polymerase (Takara Shuzo, Kyoto, Japan) using a DNA thermal cycler (Perkin-Elmer Corp., Norwalk, Connecticut, USA). The cycling conditions were: 35 cycles of 30 seconds for denaturation at 94°C, 30 seconds for annealing at 55°C, and 30 seconds for extension at 72°C, followed by a final extension at 72°C for 10 minutes. PCR products were digested with *Ssp*I (TOYOBO, Osaka, Japan), electrophoresed on a 10% polyacrylamide gel, stained with ethidium bromide, and viewed under ultraviolet light.

PCR-RFLP ANALYSIS OF *ALDH2* GENE POLYMORPHISM

ALDH2 genotyping was performed by PCR-RFLP according to the method of Harada and Misawa[21] with a slight modification. PCR was performed in a 50 μl volume of PCR mixture containing 100 ng of DNA, 10 mM Tris HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 5 pmol of each primer (sense: 5'-CAAATTACAGGGTCAAGGGCT-3'; antisense: 5'-CCACACTCACAGTTTTCTCTT-3'), 50 μM of each dNTP, and 1 U of *Taq* polymerase (Takara Shuzo, Kyoto, Japan) using the GeneAmp PCR system 9600 (Perkin-Elmer Corp.). The cycling conditions were: 35 cycles of 15 seconds for denaturation at 94°C, 45 seconds for annealing at 55°C, and 30 seconds for extension at 72°C, followed by a final extension at 72°C for 10 minutes. PCR products were digested with *Mbo*II (TOYOBO, Osaka, Japan) and separated on a 10% polyacrylamide gel.

STATISTICAL ANALYSIS

We used the Student's *t* test to test for differences among continuous variables. Fisher's exact test was also used to compare patient characteristics. Significance was assigned to

Table 1   Characteristics of head and neck cancer patients with or without multiple Lugol voiding lesions (LVL)

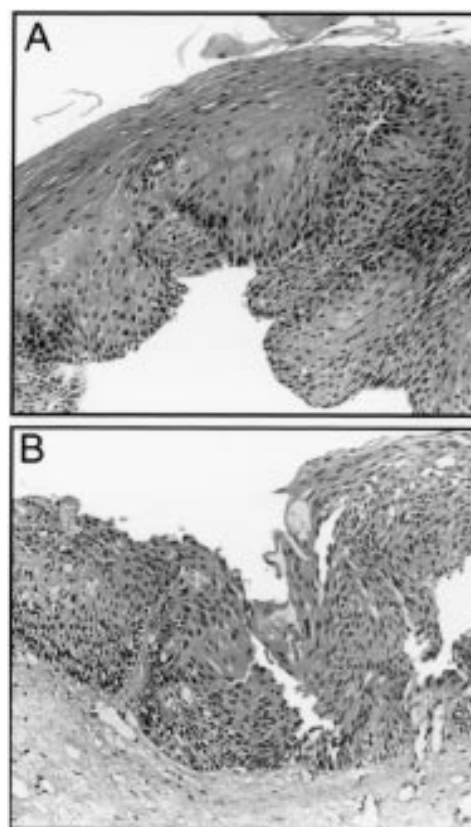|	Multiple LVL		
	Without (n=14)	With (n=17)	p Value
Sex			<0.05
Male	10	17	
Female	4	0	
Age (mean (SD))	59.8 (10.3)	65.1 (8.6)	NS
Primary tumour site of HNC			
Tongue	5	3	
Pharynx/larynx	6	9	NS
Other oral cavity	3	4	NS
Drinking habits			NS
Non-drinkers	2	2	
Drinkers	15	12	
Most often consumed drink			
Beer	3	2	
Sake (a typical Japanese liquor)	7	7	NS
Whisky	2	5	NS
Daily alcohol intake (g) (mean (SD))	60.3 (54)	68.0 (51)	
Smoking habits			<0.05
Non-smokers	4	0	
Smokers	10	17	
Brinkman index			
0	4	0	
0 to ⩽400	0	2	NS
400<	10	15	NS
Family history of cancer			NS
Present	5	9	
Absent	9	8	
Second primary oesophageal cancer			<0.0001
Present	1	14	
Absent	13	3	

Figure 2   Histological examination of multiple Lugol voiding lesions (LVL). The biopsy specimen taken from some of the multiple LVL showed severe dysplasia (A). The endoscopically resected specimen of the largest LVL in fig 1B revealed squamous cell carcinoma in situ (B).

values of $p<0.05$. Associations between putative risk factors and multiple LVL were assessed as odds ratios (OR).

Results

CLINICOPATHOLOGIAL FEATURES OF MULTIPLE LVL

Among the consecutive 31 head and neck cancer patients, 17 showed a large number of well defined irregular shaped Lugol unstained lesions throughout the oesophageal mucosa after application of Lugol dye solution (fig 1B, D). These lesions were difficult to detect on conventional endoscopic examination (fig 1A, C). All 17 patients showed similar endoscopic findings and were defined as having multiple LVL. In contrast, definite Lugol unstained lesions were hardly detected in the remaining 14 patients with head and neck cancer and these patients were defined as not having multiple LVL. Accordingly, we divided our 31 head and neck cancer patients into two groups. There were no differences in sex, age, or primary site of the head and neck cancer between these two groups (table 1).

On histological examination, several biopsy specimens of Lugol unstained lesions taken from all patients with multiple LVL showed severe dysplasia (fig 2A). The large Lugol unstained lesion in fig 1B was diagnosed as a squamous cell carcinoma in situ (fig 2B).

ASSOCIATION OF MULTIPLE LVL WITH THE SECOND PRIMARY OESOPHAGEAL CANCER IN HEAD AND NECK CANCER PATIENTS

To clarify the significance of multiple LVL in head and neck cancer patients, we examined its association with the second primary oesophageal cancer (table 1). The second primary oesophageal cancer was found in 82% (14/17) of head and neck cancer patients with multiple LVL whereas only 7% (1/14) of those without multiple LVL had oesophageal cancer ($p<0.0001$; OR 60.7, 95% CI 5.6–659.3). Furthermore, three of the head and neck cancer patients with multiple LVL had double oesophageal cancer and more than half of the oesophageal cancers concomitant with head and neck cancer were superficial carcinomas (73%; 11/15). This strong association of multiple LVL with the second primary oesophageal cancer suggests that multiple cancerous changes occurred throughout the mucosa of the upper aerodigestive tract in some patients with head and neck cancer.

ASSOCIATION OF GENETIC POLYMORPHISMS OF ALCOHOL METABOLISING ENZYMES WITH MULTIPLE LVL

To assess possible genetic and environmental factors responsible for the extensive mucosal changes in the oesophagus, we first compared drinking habits between head and neck cancer patients with multiple LVL and those without (table 1). There were no differences in drinking habits (daily alcohol consumption and type of alcoholic beverage) between the two groups. Almost all patients in both groups were defined as drinkers, drinking more than five days a week. To clarify this point further in terms of alcohol metabolism, we examined the associations of *ADH3* and *ALDH2* gene polymorphism with multiple LVL.
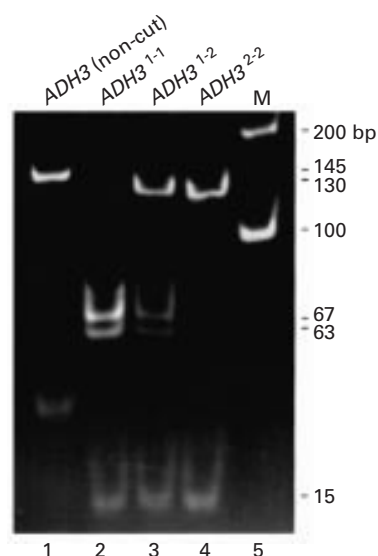
Figure 3 Representative electrophoretic patterns of the ADH3 genotype analysed by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) from three patients with the $ADH3^{1-1}$, $ADH3^{1-2}$, and $ADH3^{2-2}$ genotypes. The $ADH3^1$ allele yielded 67 bp, 63 bp, and 15 bp fragments, and the $ADH3^2$ allele appeared as a 130 bp and a 15 bp fragment. The undigested PCR product for ADH3 (ADH3 non-cut) is shown for comparison (lane 1). A 100 bp marker ladder served as a reference for DNA fragment size (M, lane 5).

The *ADH3* gene polymorphism was divided into two groups: individuals homozygous for the fast metabolising *ADH3*[1] allele ($ADH3^{1-1}$) and those who had the slow metabolising *ADH3*[2] allele ($ADH3^{1-2}$ or $ADH3^{2-2}$). PCR-RFLP analysis discriminated between the following genotypes: $ADH3^{1-1}$, $ADH3^{1-2}$, and $ADH3^{2-2}$, as shown in fig 3 (lanes 2, 3, and 4, respectively). The frequency of the slow metabolising *ADH3*[2] allele in this study was 12.9% (4/31) which was slightly higher than a previous report of 5% in Japanese subjects.[22] However, no difference was observed in the allelic frequency of the *ADH3* gene between head and neck cancer patients with and without multiple LVL (table 2).

PCR-RFLP analysis of the *ALDH2* polymorphism was performed and patients were divided into two groups based on their enzymatic activity: those homozygous for the active alleles (*ALDH2-1/2-1*) and those with an inactive allele (*ALDH2-1/2-2* or *ALDH2-2/2-2*) (fig 4). Fifteen of 31 head and neck cancer patients (48%) had the mutant *ALDH2* allele. The allelic frequency of the mutant *ALDH2* allele in head and neck cancer patients was comparable with that in the normal Japanese population.[21–24] All patients,
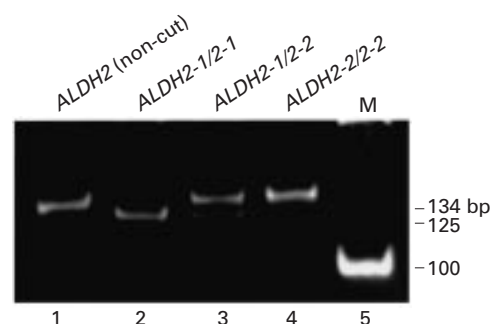

Figure 4 Representative electrophoretic pattern of the ALDH2 genotype analysed by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) from three patients with ALDH2-1/2-1, ALDH2-1/2-2, and ALDH2-2/2-2 genotypes. The ALDH2-1 allele yielded a 125 bp fragment and the ALDH2-2 allele appeared as a 134 bp fragment. The undigested PCR product for ALDH2 (ALDH2 non-cut) is shown for comparison (lane 1). A 100 bp marker ladder served as a reference for DNA fragment size (M, lane 5).

except for one with a mutant *ALDH2* allele, were heterozygotes.

Sixty five per cent (11/17) of patients with multiple LVL had the mutant *ALDH2* allele whereas 71% (10/14) of those without multiple LVL were *ALDH2-1* homozygotes (p<0.05; OR 4.6, 95% CI 1.0–21.1) (table 2). These results indicate that a mutant *ALDH2* allele is significantly associated with multiple LVL in head and neck cancer patients.

ASSOCIATION OF OTHER VARIABLES WITH MULTIPLE LVL

All 17 patients with multiple LVL were male smokers whereas the 14 patients without multiple LVL included four non-smokers and four women. To discriminate between the influence of smoking habit and sex predominance in this analysis, we also calculated ORs for multiple LVL in patients with the mutant *ALDH2* gene using the subgroups smokers and males. The observed ORs in smokers and males were 4.3 (95% CI 0.8–22.9) and 7.3 (95% CI 1.2–46.2), respectively, and were similar to the OR in all patients, as described above.

To determine the involvement of other genetic factors in the development of multiple LVL, we compared the family history of cancer between patients with and without multiple LVL (table 1). The incidence of a family history of cancer was not different between the two groups. Hence we can partly exclude the possibility that patients with multiple LVL have germline or somatic mutations in oncogenes or antioncogenes.

Discussion

In this study we assessed the correlation between multiple LVL and genetic polymorphism of alcohol metabolising enzymes in patients with head and neck cancer. The prevalence of the mutant *ALDH2* allele in patients with multiple LVL was greater than in those without multiple LVL. However, there was no significant association with the *ADH3* polymorphism, in contrast with that reported previously in Western populations in head and neck cancer.[15]

Table 2 Genetic polymorphisms of alcohol metabolising enzymes and multiple Lugol voiding lesions (LVL) in head and neck cancer patients

	Multiple LVL				
	Without	*With*	*p Value*	*OR*	*95% CI*
ADH3 polymorphism					
ADH3[1-1]	12	15			
ADH3[1-2] or ADH3[2-2]	2	2	0.622	0.8	0.1–6.5
ALDH2 polymorphism					
ALDH2-1/2-1	10	6			
ALDH2-1/2-2 or ALDH2-2/2-2	4	11	<0.05	4.6	1.0–21.1

ADH3, alcohol dehydrogenase 3; ALDH2, aldehyde dehydrogenase 2.
OR, odds ratio; 95% CI, 95% confidence interval.

In head and neck cancer patients, we frequently observed multiple LVL in the oesophagus using the endoscopic Lugol staining method. Our study showed that 17 of 31 head and neck cancer patients (55%) had multiple LVL in the oesophagus. Shiozaki *et al* also reported the presence of multiple oesophageal Lugol unstained lesions in head and neck cancer patients.[19] Furthermore, in our study, patients with multiple LVL, which consist mainly of squamous epithelial dysplasia, frequently had a second primary oesophageal cancer. As squamous epithelial dysplasia is considered a premalignant lesion of squamous cell carcinoma,[1–3] it is highly likely that the presence of multiple LVL is a predisposing condition for the development of a second primary oesophageal cancer in patients with head and neck cancer. Therefore, the presence of multiple LVL strongly suggests that genetic and/or environmental factors provoke these premalignant and malignant changes throughout the oesophageal mucosa in head and neck cancer patients.[25]

Nakanishi *et al* showed that alcohol consumption was correlated with a higher incidence of small Lugol unstained lesions in the mucosa surrounding resected oesophageal cancer.[5] Our limited study indicated that having the mutated *ALDH2* allele, rather than the amount of alcohol consumed per se, was more strongly associated with multiple LVL in head and neck cancer patients. ALDH2 is responsible for eliminating most of the acetaldehyde produced during metabolism of alcohol. Even in *ALDH2-1/2-2* heterozygotes, blood acetaldehyde concentrations after drinking are approximately six times greater than those in *ALDH2-1/2-1* homozygotes.[26] As acetaldehyde is a carcinogen[12] that induces nasopharyngeal cancer in experimental animal models,[27] this raises the possibility that severe systemic acetaldehydaemia may predispose the entire mucosa of the oesophagus to develop multiple LVL.

However, it is still not known why systemic accumulation of acetaldehyde is associated with cancer of the oesophagus but not other organs, such as the cervix. One possibility is that local ADH and ALDH activities may influence regional concentrations of acetaldehyde. One study demonstrated high ADH and low ALDH activities in the oesophageal mucosa.[28] This suggests that intracellular acetaldehyde may accumulate locally during alcohol ingestion. This may be one reason why the incidence of squamous cell carcinoma in other tissues did not increase after consumption of alcohol. Further studies are needed to elucidate the contribution of local and systemic acetaldehyde accumulation to the development of multiple LVL.

The effect of smoking on the development of multiple LVL in patients with head and neck cancer remains inconclusive as almost all patients in this study smoked. Even when non-smokers were excluded the statistical analysis showed negligible differences. Therefore, in this analysis, smoking did not affect the association between the mutant *ALDH2* allele and multiple LVL, while smoking is a definite risk factor for multiple occurrence of cancers in the upper aerodigestive tract.[10] A further large scale study is needed to elucidate this issue.

In conclusion, our findings indicate that the mutant *ALDH2* allele is closely associated with the development of multiple LVL in head and neck cancer patients. It would be interesting to determine if multiple LVL are observed in Western populations that lack the mutant *ALDH2* allele. This would help us to understand to what extent the mutant *ALDH2* allele is responsible for the development of multiple LVL.

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