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EXPRESSION OF ALDEHYDE DEHYDROGENASE 2 IN THE NORMAL ESOPHAGEAL EPITHELIUM AND ALCOHOL CONSUMPTION IN PATIENTS WITH ESOPHAGEAL CANCER

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1. ABSTRACT

Alcohol consumption is a risk factor for esophageal cancer. Acetaldehyde, a highly toxic intermediate produced from ethanol, is converted to acetic acid mainly by aldehyde dehydrogenase 2 (ALDH2) in the metabolic pathway of ethanol. Fifty percent of Japanese have inactive ALDH2 due to genetic polymorphism, which is considered to be a risk factor associated with esophageal cancer. In our previous study, we have demonstrated that ALDH2 is expressed in the esophagus with a considerable variation among individuals. In this study, we further investigated the expression of ALDH2 in esophagus and its relationship with risk factors of esophageal cancer. Tissue specimens resected from 51 patients with esophageal cancer were analyzed by immunohistochemistry using ALDH2-antibody. The immuno-staining of ALDH2 in the esophageal epithelium was compared with both the drinking habit and the occurrence of flushing that is closely associated with the ALDH2 deficiency. ALDH2 was not detectable in 8 (16%) among 51 specimens. All of the 8 patients were non- or light-drinkers but not heavy-drinkers. Among 18 patients showing the high level ALDH2 expression in the esophagus, 15 patients (83%) were heavy-drinkers. Although the relationship between the ALDH2 deficiency and drinking habit is not clear, the patients with ALDH2 deficiency tend to be non- or light drinkers while heavy-drinkers tend to have the active form of ALDH2. These results suggest that both inactive and active forms of ALDH2 are induced in the esophagus by heavy drinking and also support a hypothesis that ALDH2 deficiency might be a high-risk factor of esophageal cancer for the individuals having a heavy-drinking habit. To our knowledge, this is the first study demonstrating the induction of ALDH2 in the esophagus by ethanol consumption.

2. INTRODUCTION

Both alcohol consumption and smoking are considered to be significant risk factors for squamous cell carcinoma of the esophagus (1,2). We previously reported that these two factors have a synergic effect on the occurrence of esophageal cancer (3) and that especially heavy drinking as well as heavy smoking are closely related to the multiple occurrence of carcinomas in the upper aerodigestive tract (4-6).

Aldehyde dehydrogenase 2 (ALDH2) encoded by *ALDH2*1* (8,9) is a major enzyme for detoxification of acetaldehyde, a toxic intermediate, formed in the ethanol metabolism in humans (7). The occurrence of the genetic polymorphism of ALDH2 varies widely depending on race. The *ALDH2*2* allele having a single base substitution causes an amino acid substitution (E487K), resulting in the production of an inactive subunit. Because ALDH2 functions as a homotetramer, the inactive subunit produced by the *ALDH2*2* allele acts in a dominant negative fashion. The *ALDH2*2* allele has not been found in Caucasians or African-Americans, but it is prevalent in some Asian populations. For example, approximately 50 % of the Japanese population has the inactive phenotype (10). Persons who have inactive phenotype of ALDH2 exhibit a so-called flushing response that includes flushing, tachycardia, headache, and other unpleasant symptoms after consuming alcohol (11).

Studies of Japanese male alcoholics have shown the inactive form of ALDH2 encoded by the *ALDH2*1/2*2* gene to be a risk factor for esophageal cancer as well as oropharyngeal cancer. In addition, inactive ALDH2 has also been related to the multiple occurrence of carcinoma in the upper aerodigestive tract (12-14). However, the mechanism of the carcinogenesis of the esophagus regarding with the status of ALDH2 has remained obscure. Furthermore, the expression of ALDH2 in normal esophageal epithelium and cancerous lesions has not been well evaluated. We recently examined the tissue-distribution of both ALDH1 and ALDH2 in humans, wild mice, and ALDH2 knock-out mice. In our report (15), we demonstrated that ALDH 2 is expressed in the human esophagus as well as in the liver, and that the expression level of ALDH2 in the human esophagus varies among individuals. In this study, therefore, we investigated whether the expression level of ALDH2 in the normal esophageal epithelium is associated with the drinking status and the inactive phenotype caused by the *ALDH2*2* allele using specimens from patients with esophageal cancer.

3. MATERIALS AND METHODS

From 1993 to 2003, the esophagectomy was performed on 56 patients with squamous cell carcinoma of the esophagus at the Second Department of Surgery, University of Occupational and Environmental Health, Kitakyushu-City, Japan. Among these patients, preoperative radiation was performed for 5 patients. After excluding these patients, this study was carried out based on 51 consecutive patients with squamous cell carcinoma of the esophagus who had never received preoperative radiation.

All cases were retrospectively assessed from the medical records in the Registry of Esophageal Cancer at the University of Occupational and Environmental Health hospital. To define the alcohol drinking habit, we used the same questionnaire for all patients, as described in previous reports (4,5): The drinking status, the type of alcoholic beverage, the quantity of alcohol per week, the age when first started to drink, and for former drinkers, the age at quitting drinking were included. Taking into account the different alcohol concentrations, one drink was defined to correspond to 180 ml of sake (rice wine, most popular alcoholic beverage in Japan), 120 ml of white liquor (shochu), 70 ml of whisky, and 720 ml of beer. In terms of the accumulated amount of alcohol, the drinking index (DI="drinks/wk X yr") was defined as "number of drinks per week X number of years of drinking". The cases whose DIs were 800 or more were defined as "heavy drinkers".

Esophageal samples underwent immunohistochemical staining with the avidin-biotin complex (ABC) method using a labeled streptavidine-biotin-related antibody (LSAB) kit, as previously described (15). Briefly, the paraffin-embedded blocks of surgically resected specimens, which were proved to include both normal esophageal epithelium and carcinoma, were selected and they were cut into slices 4 μm in thickness. To investigate the expression of ALDH2, we used rabbit polyclonal antibody against human ALDH2, which was a kind gift of Dr Weiner H., University of Purdue (15, 17). The sections were dewaxed in xylene and rehydrated with ethanol. The deparaffinized sections were autoclaved at 121 degrees in citrate-buffer for 10 min. These sections were then incubated with 3 % hydrogen peroxide in methanol for 5 min to block any endogenous peroxidase activity. Protein blocking serum (DAKO: 0.25 % Casein carrier protein and NaN3) was applied for 20 min to reduce any non-specific antibody bindings. A polyclonal antibody against ALDH2 with dilution of 1: 2000 was incubated for 40 minutes at room temperature, followed by 10 minutes of incubation with biotinylated anti-rabbit immunoglobulin. The sections were then washed with phosphate-buffered saline and covered with peroxidase-conjugated streptavidin. The peroxidase activity was developed with diaminobenzidine as a chromogen for 5 min. Mayer's hematoxylin solution was then used for counter staining. The expression level of ALDH2 was independently determined by two of the authors (M.M., N.O.). Regarding the statistical analysis, either Fisher's exact test or the unpaired t test was used. A value of >0.05 was regarded as not significant.

4. RESULTS

4.1. Expression of ALDH2 in the normal squamous epithelium and clinicopathologic factors

Positive staining of ALDH2 was observed in the normal squamous epithelium of 43 patients (84 %). The expression of ALDH2 was mainly observed in the cytoplasm of the basal zone in the squamous epithelium.

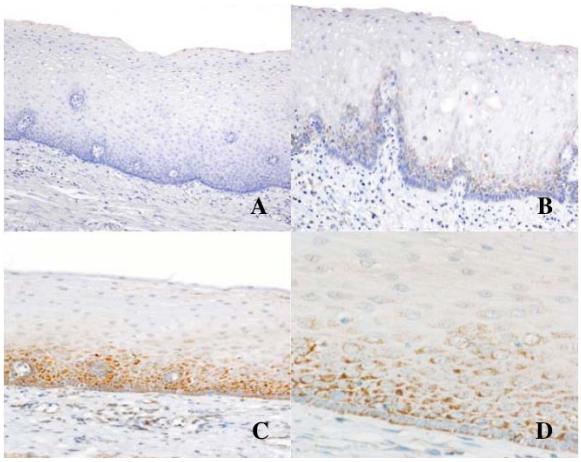

Figure 1. Expression of ALDH2 in the normal esophageal epithelium. The cases were classified according to the degree of staining in the basal one-third of the squamous epithelium. The panel A shows a representative staining image of the non-expression group whose immuno-reactivity for ALDH2 was completely negative. The panel B, the low expression group. In the image, 1-19 % of cells showed positive immuno-staining. The Panel C, the high expression group. In the image, 20% or more cells were immuno-reactive (X 100). The panel D, a high power view of the high expression group. Positive staining of ALDH2 was observed mainly in the cytoplasm of the epithelial cells in the basal zones (X 400).

According to the degree of staining in the basal one-third of the normal squamous epithelium, the cases were classified into three groups as follows: (1) A high expression group, 20 % or more epithelial cells were immuno-reactive; (2) A low expression group, 1-19 % of cells showed positive immuno-staining; and (3) A non-expression group, the immuno-reactivity for ALDH2 was completely negative (Figure 1). Eight patients (16 %) showed no expression of ALDH2, while 21 patients (41%) belonged to the high expression group. Between the two groups, no statistically significant differences were recognized regarding the clinicopahthologic factors such as age, sex, location of main tumor, differentiation of squamous cell carcinoma, invasion to the adventitia, and lymph node metastasis (Table 1).

4.2. Expression of ALDH2 and drinking habits

The relationship between the expression of ALDH2 of the normal esophageal epithelium and the drinking index was shown in Table 2. The expression of ALDH2 was closely related to the drinking habit. Two (25 %) of 8 patients who had no expression had never been alcohol drinkers, while all patients from the high expression group of ALDH2 had experience as alcohol drinkers. Regarding the amount of consumed alcoholic beverages, 17 (81 %) of 21 high expression patients were heavy drinkers whose drinking indexes were 800 or more, whereas the incidence of heavy drinkers was 27 % in the low expression group ($P<0.01$). Furthermore, no heavy drinkers were recognized in the non-expression group ($P<0.01$, compared with the high expression group).

4.3. Expression of ALDH2 and a history of flushing

Among 36 patients who underwent the interview regarding their history of flushing, 16 patients had experienced flushing after alcohol drinking. Heavy drinkers were more frequently observed among those who had never experienced flushing (60 %) than in those with a history of flushing (31 %), although the difference was not statistically significant ($P=0.11$, Table 3).

The relationship between the expression of ALDH2 in the normal esophageal epithelium and flushing in the 36 patients is shown in Table 4. A high expression of ALDH2 was recognized in 15 (75 %) among the 20 patients who had never experienced flushing, while it was only seen in 3 patients (19 %) among the 16 with a history of flushing ($P<0.01$). However, 3 patients experienced flushing in the high expression group and 5 patients had never shown any flushing in the non- or low expression groups. In order to analyze the factors influencing the expression of ALDH2 in detail, we evaluated the drinking status in each group based on both a history of flushing and the expression of ALDH2.

The frequency of non- or light-drinkers having the drinking index of less than 400 was shown in Table 5. The frequency was only 6 % in the group of high ALDH2 expression, while it was 56 % in the non- or low ALDH2 expression groups. Especially, among the 5 patients who showed either the non-expression or low expression of ALDH2, , 4 patients (80 %) were either non- or light drinkers although they never have previously experienced flushing, and the drinking index of the other 1 patient was 420. Table 6 shows the frequency of heavy drinkers whose drinking index was 800 or more. The frequency was only 11 % in the non- or low expression groups, while it was 83 % in the high ALDH2 expression group. In particular, 3 patients who showed a high ALDH2 expression were all heavy drinkers with extremely high values of drinking indices (1715, 1960, and 2940, respectively) even though they had a history of flushing.

5. DISCUSSION

Alcohol drinking and smoking are significant risk factors for almost all kinds of squamous cell carcinoma in the upper aerodigestive tract including esophageal cancer (1,2). These two factors are also closely related to the occurrence of esophageal epithelial dysplasia, which is considered to be precancerous lesion (13). Furthermore, we previously reported the multiple occurrence of squamous cell carcinoma of esophagus to be closely related to the multiplicity of dysplastic lesions (18) while the excessive exposure to alcoholic beverages and cigarettes may induce the multiple occurrence of carcinoma (4-6). Regarding a molecular analysis of alcohol-inducing carcinogenesis, we examined the expression of p53 proteins in patients with esophageal cancer, and frequently observed an abnormal expression of p53 protein in heavy drinkers as well as heavy smokers (19). On the other hand, Mori *et al.* emphasized that tumor suppressor genes, FHIT, is also one of the candidates of the target gene in the alcohol- and smoking-induced carcinogenesis of the esophagus (20).

Table 1. Expression of ALDH2 in the normal esophageal epithelium and clinicopathologic factors

	Expression of ALDH2		
Clinicopathologic factors	None	Low	High
	n=8	n=22	n=21
Age (Mean ± SE)	62.6 ± 2.0	64.6 ± 1.8	65.7 ± 1.4
Sex			
Male	7	20	20
Female	1	2	1
Location of main tumor			
Upper esophagus	0	4	3
Mid-esophagus	6	12	15
Lower esophagus	2	6	3
Differentiation of squamous cell carcinoma			
Well	4	4	2
Moderately	2	15	17
Poorly	2	3	2
Invasion to adventitia			
Negative	6	11	13
Positive	2	11	8
Lymph node metastasis			
Negative	6	10	8
Positive	2	12	13

Table 2. Expression of ALDH2 in the normal esophageal epithelium and the drinking index of patients with esophageal cancer

Drinking Index [1]	**Expression of ALDH2**					
No. of drinks/wk x yeas	None		Low		High	
	n=8		n=22		n=21	
0	2	25	3	14	0	
>0, <800	6	75	13	59	4	19
≥800	0		6	27	17	81

Numbers in parentheses are percentages, [1] Drinking Index: number of drinks per week x number of years of drinking. P<0.01 compared with both the non-expression group and the low expression group by Fisher's direct method.

Table 3. A history of flushing and the drinking index in patients with esophageal cancer

Drinking Index [a]	History of flushing			
No. of drinks/wk x years	Ever		Never	
	n=16		n=20	
0	2	13	2	10
>0, <800	9	56	6	30
≥800	5	31	12	60

Numbers in parentheses indicate percentages.Drinking Index: number of drinks per week x number of years of drinking.

Table 4. Expression of ALDH2 in the normal esophageal epithelium and flushing in patients with esophageal cancer

	Expression of ALDH2			
Flushing n	None or Low		High	
Ever 16	13	81	3	19
Never 20	5	25	15	75

□Numbers in parentheses indicate percentages. □P<0.01 by Fisher's exact test

Table 5. Frequency of non- or light-drinkers drinking index < 400

	No. of non- or light-drinker[1] / No. of cases %					
Flushing	Expression of ALDH2					
	None or Low		High		Total	
Ever	6/13	46	0/ 3	0	6/16	38
Never	4/ 5	80	1/15	7	5/20	25
Total	10/18	56	1/18	6	11/36	31

Analysis was based on both the expression of ALDH2 in the normal esophageal epithelium and the history of flushing. [1] Non- or light drinkers indicate cases whose drinking index is less than 400.

Table 6. Frequency of heavy drinkers drinking index ≥ 800

	No. of heavy drinker[1]/ No. of cases%					
Flushing	Expression of ALDH2					
	None or Low		High		Total	
Ever	2/ 13	15	3/ 3	100	6/16	31
Never	0/ 5	80	12/ 15	80	12/20	60
Total	2/18	11	15/18	83	17/36	47

Analysis was based on both the expression of ALDH2 in the normal esophageal epithelium and the history of flushing. [1] Heavy drinkers indicate cases whose drinking index is 800 or more.

Yokoyama *et al.* (12,13) examined the genetic polymorphisms of ALDH2 in Japanese alcoholics. As a result, they found the mutant ALDH2 allele to be a risk factor of the occurrence of esophageal cancer while it was also a risk factor for multiple UADT cancers. Among Chinese alcoholics, mutant ALDH2 also proved to be a risk factor for esophageal cancer (21). When considering the role of ALDH2 in esophageal carcinogenesis, it is important to examine the expression of ALDH2 of the esophagus. This study is the first report, in which the expression of ALDH2 in the esophagus was immuno-histochemically examined. We also examined the relationship between the expression of this enzyme and the drinking status as well as the history of flushing. As a result, ALDH2 was expressed in the normal esophageal epithelium although the degree of expression varied from case to case. The expression of ALDH2 was strongly related to the accumulated quantity of the alcoholic beverage. These results clearly suggest that exposure to alcohol may induce the topical expression of ALDH2 in the esophageal epithelium.

Regarding the screening for this inactive ALDH2 phenotype, the questionnaire for so-called flushing has been shown to be reliable. The sensitivity and specificity for identifying inactive ALDH2 were reported to be 96.1 % and 71.4 %, respectively (16). In this study, a low level expression of ALDH2 in the esophageal epithelium was frequently observed in patients with a history of flushing, which thus reflects the presence of inactive ALDH2. Furthermore, those who show a high expression of ALDH2 and have history of flushing were all extremely heavy drinkers. On the other hand, among those who were considered to have active ALDH2 (patients having no experience of flushing), a high level of expression was frequently observed, while most cases showing a low level expression were either non- or light drinkers. These results suggest that the exposure to alcoholic beverage strongly appears to be related to the expression of ALDH2 in the esophagus regardless of the genetic status of ALDH2.

Yin *et al.* (22) reported the esophageal ALDH activity to be only 20 % of that in the stomach and they discussed that low level ALDH might induce the local accumulation of acetaldehyde, which might thus result in esophageal carcinogenesis. On the other hand, our current study revealed that ALDH2 could be induced in the esophagus even in those with a history of flushing. However, in such patients, the degree of ALDH2 expression was generally low. A high expression was only observed when they had consumed extremely large amounts of alcohol. These results present a possibility that, in those who have the mutant ALDH2 allele, a low level expression of ALDH2 and/or an excessive consumption of alcoholic beverages might cause the accumulation of acetaldehyde in the esophageal epithelium. As a result, the local accumulation of acetaldehyde might thus be related to the high frequency of esophageal cancer in such individuals.

6. CONCLUSIONS

The genetic status of ALDH2 as well as alcohol consumption strongly appears to be important in the expression of ALDH2 in the esophagus. The high frequency of esophageal cancer in those who have inactive ALDH2 may be related to a low level of the expression of ALDH2 and/or excessive alcohol consumption.

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Abbreviations: ALDH, Aldehyde dehydrogenase; DI, drinking index; UADT, upper aerodigestive tract; FHIT, fragile histidine triad

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