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Establishment of a Quick and Highly Accurate Breath Test for ALDH2 Genotyping

Ikuo Aoyama, MD[1], Shinya Ohashi, MD, PhD[1], Yusuke Amanuma, MD, PhD[1], Kenshiro Hirohashi, MD[2], Ayaka Mizumoto, MS[1], Makiko Funakoshi, MD[1], Mihoko Tsurumaki, MS[1], Yukie Nakai, MS[1], Katsuyuki Tanaka, PhD[3], Mariko Hanada, BS[3], Aki Uesaka, BS[3], Tsutomu Chiba, MD, PhD[2] and Manabu Muto, MD, PhD[1]

OBJECTIVES: Acetaldehyde, the first metabolite of ethanol, is a definite carcinogen for the esophagus, head, and neck; and aldehyde dehydrogenase 2 (ALDH2) is a mitochondrial enzyme that catalyzes the metabolism of acetaldehyde. The *ALDH2* genotype exists as *ALDH2*1/*1* (active ALDH2), *ALDH2*1/*2* (heterozygous inactive ALDH2), and *ALDH2*2/*2* (homozygous inactive ALDH2). Many epidemiological studies have reported that *ALDH2*2* carriers are at high risk for esophageal or head and neck squamous cell carcinomas by habitual drinking. Therefore, identification of *ALDH2*2* carriers would be helpful for the prevention of those cancers, but there have been no methods suitable for mass screening to identify these individuals.

METHODS: One hundred and eleven healthy volunteers (*ALDH2*1/*1* carriers: 53; *ALDH2*1/*2* carriers: 48; and *ALDH2*2/*2* carriers: 10) were recruited. Breath samples were collected after drinking 100 ml of 0.5% ethanol using specially designed gas bags, and breath ethanol and acetaldehyde levels were measured by semiconductor gas chromatography.

RESULTS: The median (range) breath acetaldehyde levels at 1 min after alcohol ingestion were 96.1 (18.1–399.0) parts per billion (p.p.b.) for the *ALDH2*1/*1* genotype, 333.5 (78.4–1218.4) p.p.b. for the *ALDH2*1/*2* genotype, and 537.1 (213.2–1353.8) p.p.b. for the *ALDH2*2/*2* genotype. The breath acetaldehyde levels in *ALDH2*2* carriers were significantly higher than for the *ALDH2*1/*1* genotype. Notably, the ratio of breath acetaldehyde level-to-breath ethanol level could identify carriers of the *ALDH2*2* allele very accurately (whole accuracy; 96.4%).

CONCLUSIONS: Our novel breath test is a useful tool for identifying *ALDH2*2* carriers, who are at high risk for esophageal and head and neck cancers.

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INTRODUCTION

Esophageal squamous cell carcinomas (ESCC) and head and neck squamous cell carcinomas (HNSCC) are some of the deadliest cancers worldwide, and acetaldehyde, the first metabolite of ethanol, is a definite carcinogen for these organs.[1–3] Acetaldehyde-induced carcinogenesis is considered to be attributable to single- and double-stranded DNA breaks, point mutations, sister chromatid exchanges, and gross chromosomal aberrations.[4–7] Acetaldehyde is generated from metabolism of ethanol mainly by alcohol dehydrogenases (ADHs) such as ADH 1B (rs1229984) which is one of the major ADHs, and then eliminated by aldehyde dehydrogenase 2 (ALDH2; rs671).[8,9] ADH1B and ALDH2 have polymorphisms that result in different activities.

ADH1B has two alleles, *ADH1B*1* (less active ADH1B) and *ADH1B*2* (active ADH1B). Therefore, there are three ADH1B genotypes: *ADH1B*1/*1*, less active slow-metabolizing ADH1B; *ADH1B*1/*2*; and *ADH1B*2/*2*, active ADH1B. The alcohol-elimination rate in those with the *ADH1B*1/*1* genotype is about 12% lower than that in *ADH1B*2* carriers (*ADH1B*1/*2* and *ADH1B*2/*2*), although there is no difference in the activity between *ADH1B*1/*2* and *ADH1B*2/*2* carriers.[10]

ALDH2 also has two alleles, *ALDH2*1* (active ALDH2) and *ALDH2*2* (inactive ALDH2). ALDH2 genotypes are classified as follows: *ALDH2*1/*1*, active ALDH2; *ALDH2*1/*2*, inactive ($<$10% activity) ALDH2; and *ALDH2*2/*2*, inactive (0% activity) ALDH2.[11–14] Carriers of the *ALDH2*2* allele (*ALDH2*1/*2* and *ALDH2*2/*2*) account for 40–50% of east-Asian populations.[15–17] The ability to metabolize acetaldehyde is very low in *ALDH2*2* carriers compared with those of genotype *ALDH2*1/*1*, so blood, salivary, and breath acetaldehyde levels are elevated when they drink alcohol.[8,18,19]

*ALDH2*2/*2* genotype individuals are usually non-drinkers or occasional drinkers because they cannot metabolize acetaldehyde; however, the inhibitory effect on drinking of being heterozygous for inactive ALDH2 (*ALDH2*1/*2*) is influenced by socio-cultural factors,[20] and some *ALDH2*1/*2* individuals tend to be heavy alcohol drinkers. Indeed, the proportion of alcoholics in Japan who have the *ALDH2*1/*2* genotype is 15.4%.[21] Of note, many epidemiological studies have

[1]Department of Therapeutic Oncology, Kyoto University, Graduate School of Medicine, Kyoto, Japan; [2]Department of Gastroenterology and Hepatology, Kyoto University, Graduate School of Medicine, Kyoto, Japan and [3]Nissha Printing Co., Ltd, Kitazono, Itami, Japan
Correspondence: Manabu Muto, MD, Department of Therapeutic Oncology, Kyoto University, Graduate School of Medicine, Kyoto 606-8507, Japan.
E-mail: mmuto@kuhp.kyoto-u.ac.jp
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demonstrated that *ALDH2*1/*2* individuals who are heavy alcohol drinkers have a high risk of ESCC and HNSCC.[20,22–25]

Therefore, identifying such individuals is very important for prevention of ESCC and HNSCC. Additionally, ALDH2 gene polymorphism is related to many alcohol-related events, e.g., alcohol use disorder,[26] hypertension,[27] ischemic heart disease,[28,29] and alcohol-induced asthma.[30] Thus effective identification of ALDH2 gene polymorphism may contribute to prevention of such diseases.

Genetic testing is the most reliable way to identify carriers of the *ALDH2*2* allele. However, it is not suitable for mass screening because it requires time and cumbersome procedure. The ethanol patch test and/or the flushing questionnaire[31,32] have been considered as alternative diagnostic tools, but their objective assessment is difficult and their accuracy is unsatisfactory.[31,32] Consequently, there has been no suitable mass screening tool for identifying carriers of the *ALDH2*2* allele.

Here we report the establishment of a new breath test that can measure very low levels of acetaldehyde in a quantitative way after ingestion of a very small amount of alcohol (100 ml of 0.5% ethanol) and that can accurately and rapidly identify carriers of the *ALDH2*2* allele.

METHODS

Participants and intervention. This study was approved by the Institutional Review Board of the Kyoto University (Review No. G626). One hundred and eleven Japanese healthy volunteers were recruited from March 2014 to March 2015. Men or women aged >20 years were eligible for inclusion if they did not have histories of gastro-intestinal surgery or liver dysfunction. After participants provided written informed consent, their demographic data were collected by self-completed questionnaire.

Participants were asked to drink 100 ml of 0.5% ethanol in one draught after at least 3 h of fasting and 12 h abstinence. The 0.5% ethanol was made with vodka, which contains little acetaldehyde.[33] Breath samples were collected with a originally established gas bag immediately before and 1, 2, and 5 min after drinking the alcohol.

Collection of end-tidal gas. We developed a new type of gas bag to collect the end-tidal gas. The gas bag is made of vinyl alcohol polymer and has a unique shape to remove the gas derived from the physiological dead space (Figure 1a). About 100 ml of end-tidal gas can be collected with one breath into the bag. In this study, the breath was collected in these bags at standard temperature under air conditioning.

Measurement of breath acetaldehyde and ethanol levels. Breath acetaldehyde and ethanol levels were measured by highly sensitive gas chromatography[34] using a Sensor Gas Chromatograph SGEA-P2 (FIS, Hyogo Japan). Exhaled gas was drawn up from the bag in a 5 ml syringe and injected into the gas chromatograph.

The conditions for analysis were as follows: the column temperature was 90 °C, the carrier gas flow rate was 100 ml/min, and the volume of gas injected was 5 ml. The measurable range of acetaldehyde and ethanol levels were as follows: acetaldehyde, 5–10,000 parts per billion (p.p.b.); ethanol, 200–100,000 p.p.b. The achievable resolution was 0.1 p.p.b. The retention times of acetaldehyde and ethanol were approximately 50 and 85 s, respectively. Including wash-out time (4 min), the measurement could be completed within 8 min. The investigators (M.H. and A.U.) who analyzed the level of acetaldehyde and ethanol were blinded to the results of the genetic testing and the information obtained from the questionnaire.

Genotyping. To determine the *ADH1B* and *ALDH2* genotypes, we isolated genomic DNA from whole blood using the QIAamp DNA Blood Midi Kit (Qiagen, Hilden, Germany). *ADH1B* genotyping was performed by a PCR–restriction fragment-length polymorphism method.[35] *ALDH2* genotyping was performed by the Smart Amplification Process.[36] The investigators (T.M. and N.Y.) who analyzed the genetic polymorphisms were blinded to the results of the breath test and the information obtained from the questionnaire.

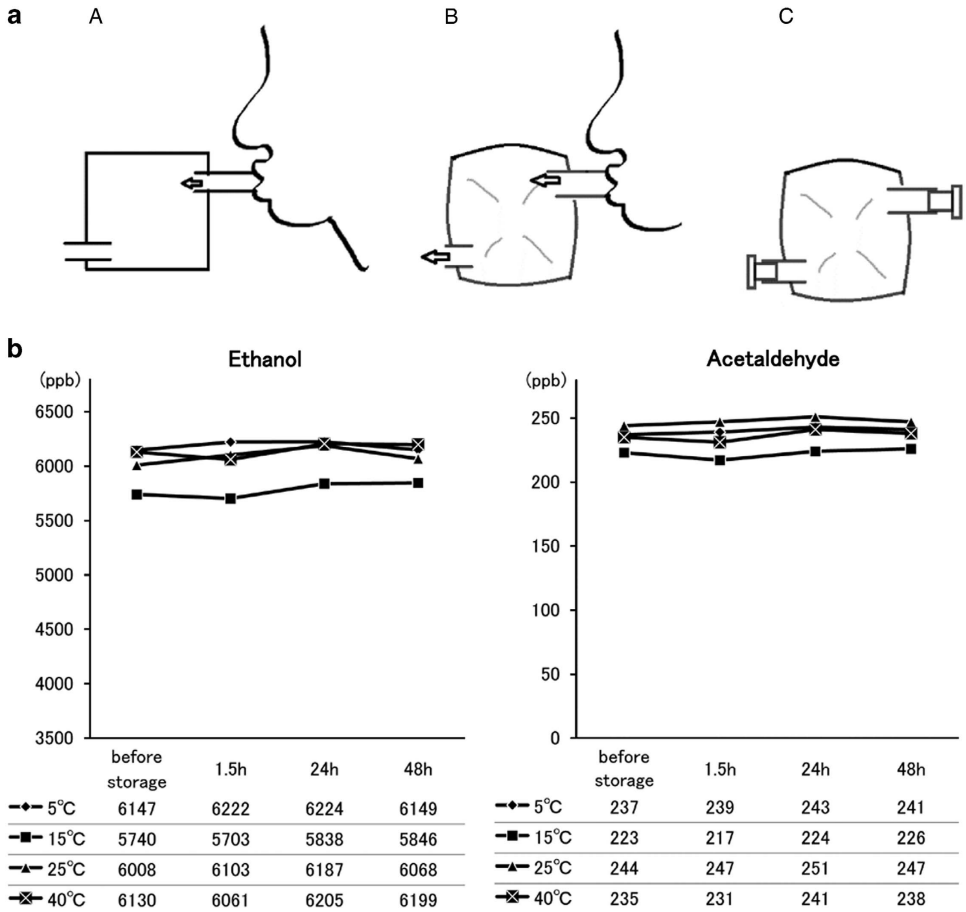
Statistical analysis. Statistical analyses were performed using the JMPPro11 software (SAS Institute, Cary, NC).

The distributions of sex, smoking habits, daily alcohol consumption, and *ADH1B* genotype in *ALDH2*1/*1* participants and carriers of the *ALDH2*2* allele were compared using Pearson's chi-square test. Wilcoxon's rank-sum test was used for comparison of age with breath acetaldehyde level and the ratio of acetaldehyde to ethanol (A/E ratio). The differences in ethanol levels between different *ADH1B* genotypes and in acetaldehyde levels between different *ALDH2* genotypes were compared using the Kruskal–Wallis test. Spearman's correlation coefficients were calculated to express the strength of the relationship between breath acetaldehyde and ethanol levels 1 min after consumption of 100 ml of 0.5% ethanol.

The diagnostic performance of the breath test and the flushing questionnaire for detecting *ALDH2*2* carriers was assessed in terms of sensitivity (the proportion of individuals truly possessing the *ALDH2*2* allele who were identified by breath test or flushing questionnaire), specificity (the proportion of individuals truly possessing *ALDH2*1/*1* who were identified by breath test or flushing questionnaire), and accuracy (the proportion of all participants truly possessing *ALDH2*2* and the proportion of individuals truly possessing *ALDH2*1/*1* who were identified by breath test or flushing questionnaire). A P value <0.05 was considered to be significant.

RESULTS

Stability of acetaldehyde and ethanol levels in the collection bag. Because acetaldehyde is highly adsorptive and thus has the potential to be adsorbed onto the surface of the gas bag, we examined the stability of acetaldehyde and ethanol levels in our gas bags. Figure 1b illustrates the stability of acetaldehyde and ethanol concentrations in a primary standard gas, which was prepared by mixing highly purified air with acetaldehyde and ethanol at concentrations of approximately 250 and 6,000 p.p.b., respectively.



	before storage	1.5h	24h	48h
5℃	6147	6222	6224	6149
15℃	5740	5703	5838	5846
25℃	6008	6103	6187	6068
40℃	6130	6061	6205	6199

	before storage	1.5h	24h	48h
5℃	237	239	243	241
15℃	223	217	224	226
25℃	244	247	251	247
40℃	235	231	241	238

Figure 1 Methods for collecting end-tidal gas and determining the stability of acetaldehyde and ethanol in the bag. (**a**) The gas collection bag. (A) The gas collection bag has an inspiratory port and an exhaust port. (B) The gas derived from the physiological dead space was removed from the exhaust port. (C) With each breath into the bag, about 100 ml of end-tidal gas can be collected. (**b**) Time-dependent changes in acetaldehyde and ethanol levels in the collected breath at various temperatures. The simulated gas was prepared by mixing highly purified air, acetaldehyde, and ethanol. The concentrations of acetaldehyde and ethanol were adjusted to be approximately 250 and 6,000 p.p.b., respectively, which were selected on the basis of the concentrations in a preliminary breath test. The simulated gas was injected into the gas bags, which were sealed and stored at various temperatures (5, 15, 25, and 40 °C). Concentrations of acetaldehyde and ethanol were measured immediately before storage and after 1.5, 24, and 48 h of storage. Levels of both acetaldehyde and ethanol were maintained within 3.0% of error range at each temperature over 48 h.

Acetaldehyde levels were maintained over 48 h within an error range of 2.9% at all temperatures. Ethanol levels were also maintained within an error range of 3.0%.

Background acetaldehyde and ethanol levels. To validate the background acetaldehyde and ethanol levels, we measured those concentrations in the collection bags. The acetaldehyde and ethanol concentrations in the air that had been filled in the bag for 1 h at 25 and/or 60 °C, respectively, was below the detection limit ($<$5 p.p.b. in acetaldehyde, $<$200 p.p.b. in ethanol) of the gas chromatography. Next, we measured the acetaldehyde level in the head space of undiluted vodka solution (40% of ethanol concentration) at room temperature (around 25 °C). Also, in this case, the acetaldehyde level was below the detection limit ($<$5 p.p.b.) of the gas chromatography.

Furthermore, we measured acetone level of end-tidal gas, which was collected immediately before and/or 1, 2, and 5 min after drinking the alcohol (0.5% ethanol, 100 ml), in the collection bags of each participant to verify the presence or absence of air contamination. The acetone levels were not significantly changed among these samples. These results indicate that external air contamination is considered not to have occurred in this collection bags.

Study population. The participants included 77 men and 34 women, with a mean age of 37.2 $\pm$ 8.8 (s.d.) years. Of the participants, 53 were *ALDH2*1/*1*, 48 were *ALDH2*1/*2*, and 10 were *ALDH2*2/*2* genotype. Their characteristics are shown in Table 1. Among the 53 participants with the *ALDH2*1/*1* genotype, 3 were *ADH1B*1/*1*, 19 were *ADH1B*1/*2*, and 31 were *ADH1B*2/*2*. Among the 58 carriers of the *ALDH2*2* allele (48 *ALDH2*1/*2*, 10 *ALDH2*2/*2*), 1 participant was *ADH1B*1/*1*, 20 were *ADH1B*1/*2*, and 37 were *ADH1B*2/*2*. There were no significant differences between the *ALDH2*1/*1* genotype carriers and carriers of the *ALDH2*2* allele (*ALDH2*1/*2* and *ALDH2*2/*2*) regarding age, smoking habits, body mass index, and distribution of the *ADH1B* genotype. However, carriers of the *ALDH2*2* allele included a significantly higher

Table 1 Characteristics of the study participants

	***ALDH2 *1/*1* (active) $n=53$**	***ALDH2 *1/*2* (less active) $n=48$**	***ALDH2 *2/*2* (inactive) $n=10$**	***P* value[a]**
Sex (male/female)	42/11	30/18	5/5	0.04
Age, median (range)	35 (25–74)	35 (26–59)	35 (22–48)	0.61
BMI, median (range)	22.0 (17.7–27.8)	22.0 (18.2–34.9)	21.0 (17.3–31.7)	0.63
Smoking (current/past/never)	8/8/37	6/6/36	0/1/9	0.63
Daily alcohol consumption >20 g ethanol/day, yes/no	21/32	7/41	0/10	0.001
Alcohol-related flushing				<0.001
Former or current flushing	6	45	9	
Never flushed	47	3	1	
ADH1B genotype				0.31
*ADH1B *1/*1* (less active)	3	1	0	
*ADH1B *1/*2*	19	15	5	
*ADH1B *2/*2* (active)	31	32	5	

BMI, body mass index.
[a]Comparisons were made between participants with *ALDH2*1/*1* genotype and all carriers of the *ALDH2*2* allele (*ALDH2*1/*2*+*ALDH2*2/*2* genotypes).

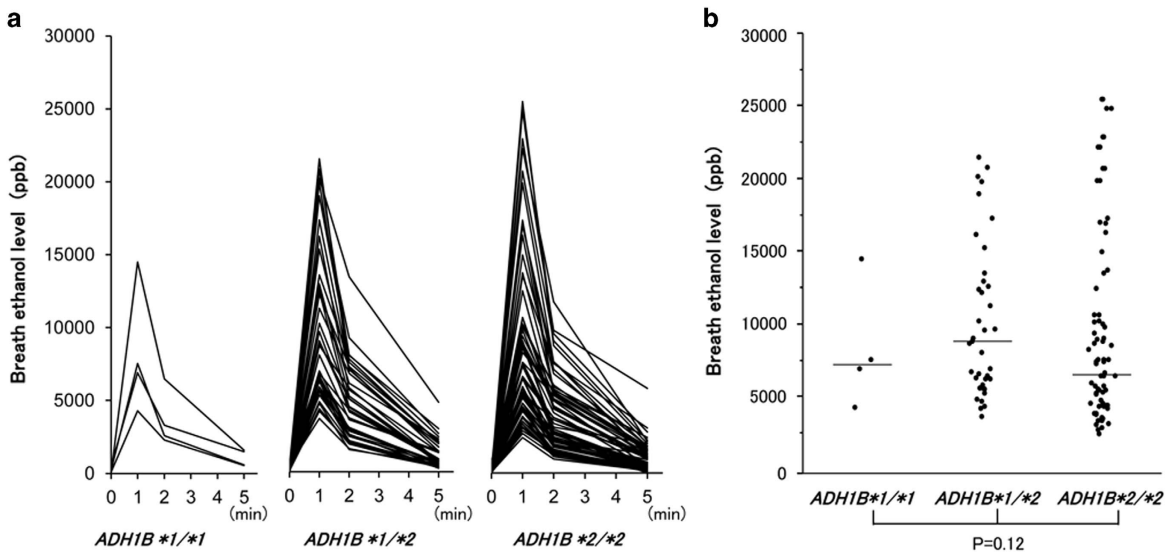


Figure 2 Breath ethanol levels in individuals with different *ADH1B* genotypes. (**a**) Time-dependent changes in breath ethanol levels according to *ADH1B* genotype. In all participants, the breath ethanol levels peaked at 1 min after alcohol ingestion and then decreased immediately. (**b**) Each ethanol level at 1 min after ethanol ingestion was plotted (horizontal line = median value). There were no differences in the ethanol levels between different *ADH1B* genotypes ($P=0.12$; Kruskal–Wallis test).

percentage of women and had a significantly lower daily alcohol consumption than participants with the *ALDH2*1/*1* genotype (Table 1).

The breath ethanol level. In all participants, the breath ethanol level was extremely low (maximum level 640 p.p.b.; 0.0012 mg/l) before alcohol ingestion, reached a peak 1 min after alcohol ingestion, and then decreased rapidly (Figure 2a). The breath ethanol level at 1 min after alcohol ingestion ranged from 25,400 p.p.b. (0.048 mg/l) to 2,430 p.p.b. There were no significant differences in breath ethanol levels between *ADH1B* genotype groups ($P=0.12$; Figure 2b).

The breath acetaldehyde level. The peak breath acetaldehyde levels were obtained 1 min after alcohol ingestion in the majority of the participants, although levels in three participants in the *ALDH2*2/**2 group showed peak levels at 2 or 5 min after ingestion (Figure 3a). The median (range) of breath acetaldehyde levels at 1 min after alcohol ingestion was 96.1 (18.1–399.0) p.p.b. in *ALDH2*1/*1* participants, 333.5 (78.4–1218.4) p.p.b. in *ALDH2*1/*2* participants, and 537.1 (213.2–1353.8) p.p.b. in *ALDH2*2/*2* participants, respectively. Breath acetaldehyde levels in *ALDH2*1/*2* or *ALDH2*2/*2* participants were significantly higher than those in *ALDH2*1/*1* participants ($P<0.001$ for both). Breath acetaldehyde levels in *ALDH2*2* participants (*ALDH2**1/*2 or *ALDH2**2/*2) were also significantly higher than those in

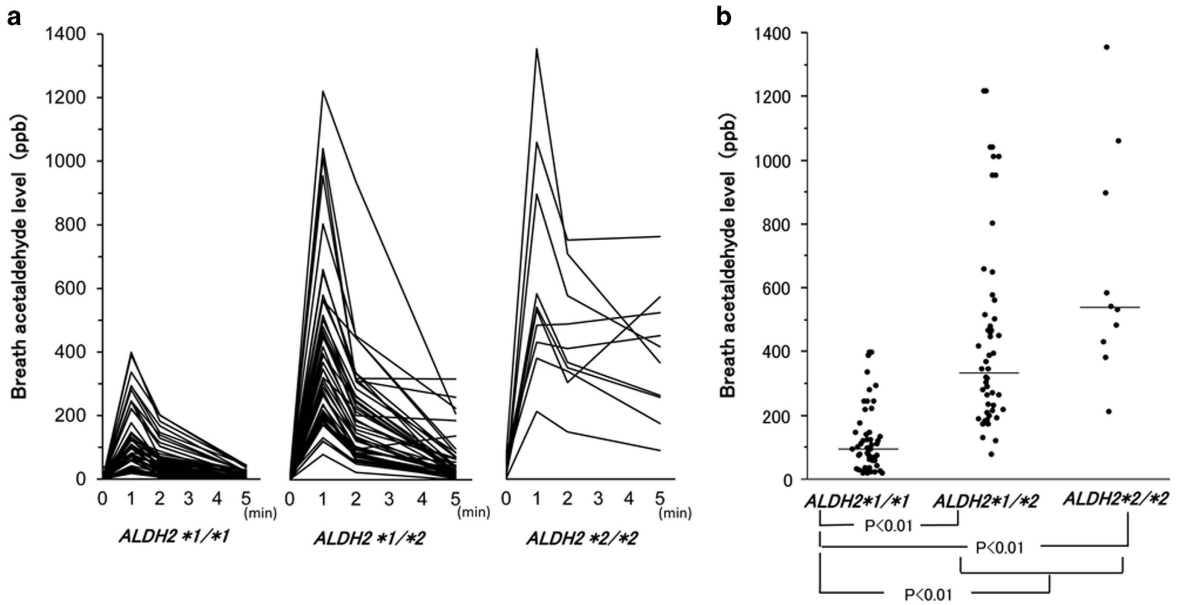


Figure 3 Breath acetaldehyde levels in individuals with different *ALDH2* genotypes. (**a**) Time-dependent changes in breath acetaldehyde levels according to *ALDH2* genotype. The majority of participants showed a peak acetaldehyde value at 1 min after alcohol ingestion, but some *ALDH2*2* carriers ($n=3$) showed their highest peaks at 2 or 5 min after ingestion. (**b**) Each acetaldehyde level at 1 min after ethanol ingestion was plotted (horizontal line = median value). Breath acetaldehyde levels in carriers of the *ALDH2*2* allele (*ALDH2*1/*2* and *ALDH2*2/*2*) were significantly higher than those in *ALDH2*1/*1* participants ($P<0.001$).

*ALDH2*1/*1* participants ($P<0.001$) (Figure 3b). There were no significant differences in breath aldehyde levels between *ADH1B* genotypes (*ADH1B**1/*1, *ADH1B*1/*2*, and *ADH1B*2/*2*; data not shown).

Notably, no participant of any *ALDH2* genotype had a flushing reaction nor nausea after ingestion of 100 ml of 0.5% ethanol. When we set the breath acetaldehyde cutoff value to 75 p.p.b. to detect the lowest breath acetaldehyde values in *ALDH2*2* allele (*ALDH2*1/*2* and *ALDH2*2/*2*) carriers, the diagnostic performance showed a specificity of 43.4% (23/53) and an accuracy of 73.0% (81/111).

Relationship between breath acetaldehyde/ethanol levels and *ALDH2*2* allele. Breath acetaldehyde levels showed relatively high individual differences even in participants with the same *ALDH2* genotype (Figure 3a). Therefore, the absolute value of the breath acetaldehyde level cannot accurately differentiate *ALDH2*2* allele carriers from *ALDH2*1/*1* carriers. We speculated that differences in the rate of ethanol absorption from the gastro-intestinal tract might have influenced the absolute acetaldehyde level in the breath. Indeed, we found that there was a strong positive correlation between breath acetaldehyde and ethanol levels 1 min after alcohol ingestion in all groups. The correlation coefficients by Spearman's rank test were 0.83 for *ALDH2*1/*1* individuals ($P<0.001$) and 0.80 for carriers of the *ALDH2*2* allele ($P<0.001$) (Figure 4). These findings prompted us to examine whether the A/E ratio could differentiate *ALDH2* genotypes more accurately. The median A/E ratio (range) at 1 min after drinking was 11.8 (4.36–39.8) in *ALDH2*1/*1* participants and 44.6 (23.4–109.4) in carriers of the *ALDH2*2* allele. The A/E ratio in carriers of the

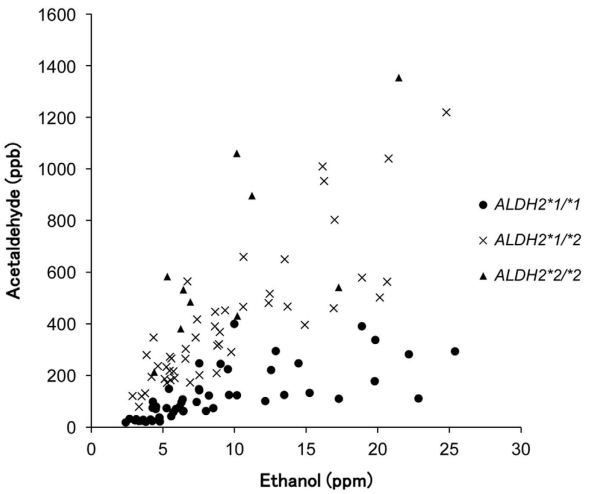


Figure 4 Relationship between acetaldehyde and ethanol levels of each individual 1 min after ethanol ingestion. Breath acetaldehyde and ethanol levels of each individual 1 min after drinking 100 ml of 0.5% ethanol are plotted. There were significant correlations between breath acetaldehyde and ethanol levels in *ALDH2*1/*1* and *ALDH2*1/*2* carriers. Correlation coefficients in Spearman's rank test were 0.83 for *ALDH2*1/*1* ($P<0.001$), 0.86 for *ALDH2*1/*2* ($P<0.001$), and 0.62 for *ALDH2*2/*2* ($P=0.06$) genotypes.

*ALDH2*2* allele was significantly higher than that in *ALDH2*1/*1* participants ($P<0.001$) (Figure 5).

To identify the factors affecting the A/E ratio, simple and multiple regression analyses were performed. By simple regression analysis, the explanatory variables of *ADH1B* genotype, age, body mass index, and smoking habits were excluded. Multiple regression analysis was performed for the

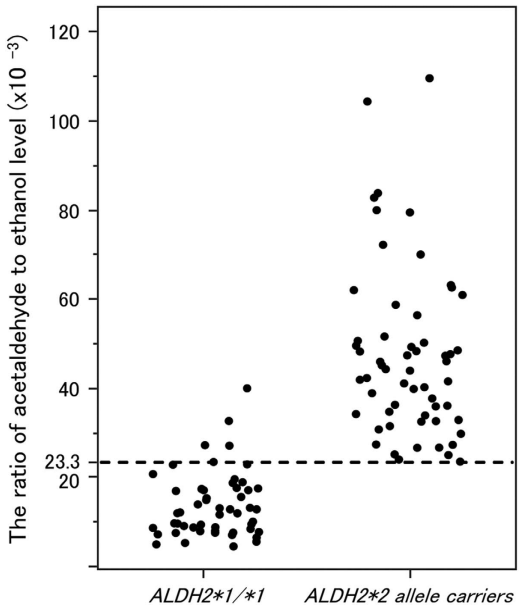


Figure 5 The ratio of acetaldehyde-to-ethanol level (A/E ratio) for each individual 1 min after ethanol ingestion. The A/E ratios of each individual 1 min after alcohol ingestion were plotted. There was a significant difference in the A/E ratios of *ALDH2*1/*1* genotype and carriers of the *ALDH2*2* allele when the cutoff value was set at 23.3.

explanatory variables of *ALDH2* genotype and sex and alcohol consumption and demonstrated that only the *ALDH2* genotype was correlated with the A/E ratio (Table 2).

We set the cutoff level for the A/E ratio at 23.3×10^{-3} so as not to overlook the lowest A/E ratio in carriers of the *ALDH2*2* allele. The accuracy, sensitivity, and specificity of the A/E ratio for identifying carriers of the *ALDH2*2* allele were 96.4% (107/111), 100% (58/58), and 92.5% (49/53), respectively (Figure 5, Table 3).

DISCUSSION

An expired gas test has been suggested to be an appropriate tool for identifying carriers of the *ALDH2*2* allele. Indeed, previous reports showed that breath acetaldehyde levels well reflected the blood acetaldehyde levels[37] and that the breath acetaldehyde levels after drinking alcohol were higher in *ALDH2*1/*2* individuals than in *ALDH2*1/*1* individuals.[18] However, the method described in this previous report was not suited for mass screening, because a high ethanol concentration (6% ethanol, 200 ml) was used.[18] In the present study, we established a quick breath test that measures breath acetaldehyde levels in a quantitative way after consumption of a very small amount of alcohol. This test could accurately identify carriers of the *ALDH2*2* allele within 8 min, thereby

Table 2 Effect of various factors on the ratio of acetaldehyde-to-ethanol level (A/E ratio)

Simple regression analysis

Variable	Standard regression coefficient (β)	*P* value
ALDH2 (*2 allele carrier)	−16.80	<0.001
ADH1B (*1/*1)	−9.96	0.192
ADH1B (*1/*2)	5.17	0.255
ADH1B (*2/*2)	4.20	0.268
Sex	5.96	0.009
Age	−2.58	0.288
Smoking (current)	−0.94	0.959
Smoking (never)	2.01	0.853
Smoking (past)	−1.06	0.537
Alcohol consumption (>20 g ethanol/day)	5.75	0.018

Multiple regression analysis

Variable	Standard partial regression coefficient (β)	VIF	*P* value	R^2	*P* value
				0.581	<0.001
ALDH2 (*2 allele carrier)	−0.74	1.142	<0.001		
Sex	0.10	1.055	0.125		
Alcohol consumption (>20 g ethanol/day)	−0.03	1.124	0.685		

VIF, variance inflation factor.

Table 3 Diagnostic ability of A/E ratio for carriers of the *ALDH2*2* allele

	Sensitivity	Specificity	Accuracy	PPV	NPV
	100% (58/58)	92.5% (49/53)	96.4% (107/111)	93.5% (58/62)	100% (49/49)
95% CI	90.9–100	81.8–97.9	91.0–99.0	84.3–98.2	89.4–100

A/E ratio, acetaldehyde-to-ethanol level ratio; CI, confidence interval; NPV, negative predictive value; PPV, positive prhedictive value.

subject will be able to know the result shortly after the test, and thus this test is useful at educational as well as clinical situations.

We showed that breath acetaldehyde levels after alcohol ingestion in carriers of the *ALDH2*2* allele were significantly higher than those in *ALDH2*1/*1* participants. However, breath acetaldehyde levels varied considerably even between participants with the same genotype, and their diagnostic performance for identifying carriers of the *ALDH2*2* allele was unsatisfactory. We hypothesized that differences in the rate of ethanol absorption from the gastro-intestinal tract might have influenced the absolute acetaldehyde level in the breath. In this regard, it is notable that we observed a strong correlation between the acetaldehyde and ethanol levels in each breath sample. This finding led us to speculate that the breath A/E ratio could predict carriers of the *ALDH2*2* allele more accurately than the acetaldehyde level itself. As predicted, we found that the A/E ratio was able to detect carriers of the *ALDH2*2* allele with high sensitivity and specificity (sensitivity: 100%, specificity: 92.5%, accuracy: 96.4%). The data clearly show that the breath A/E ratio measured by our method is a satisfactory screening marker for identifying *ALDH2* genotype. Although we could not distinguish between *ALDH2*1/*2* and *ALDH2*2/*2* genotypes by measuring the A/E ratio (data not shown), this distinction is not important clinically, because all carriers of the *ALDH2*2* allele should limit their consumption of alcohol.

We found in this study that the breath ethanol level was not influenced by the *ADH1B* genotype, although breath ethanol levels in carriers of the *ADH1B*1/*1* genotype are theoretically higher than those in carriers of the *ADH1B*2* allele (*ADH1B*1/*2* and *ADH1B*2/*2*). We assume that this is because of the limited difference in ADH1B activity between *ADH1B*1* and *ADH1B*2* alleles. The breath acetaldehyde level also was not influenced by the *ADH1B* genotype (data not shown). These results are consistent with previous reports that *ADH1B* genotype does not correlate with blood ethanol or acetaldehyde levels after ingestion of usual amounts of alcohol.[38–41]

The breath ethanol level peaked at 1 min after alcohol ingestion in all participants. The highest breath ethanol concentration was 25,400 p.p.b. (0.048 mg/l), and its levels decreased rapidly within 5 min (Figure 2). Referring to the legal breath alcohol level (0.35 mg/l) for driving in the United Kingdom,[42] this ethanol intake (0.5% ethanol, 100 ml) in our study is considered acceptable for screening. In fact, no participant felt sick or had a flushing reaction; therefore, this test is a minimally invasive tool for identifying carriers of the *ALDH2*2* allele.

This study also showed that breath acetaldehyde levels widely varied in any genotype. The difference was bigger in those with *ALDH2*2* allele than in those with *ALDH2*1/*1*. This might mean that individual difference of acetaldehyde exposure contribute to the acetaldehyde-related carcinogenesis in the esophagus and head and neck region even in the case of same amount of alcohol ingestion.

This study had some limitations. It was conducted using relatively young people and light drinkers, although esophageal cancer is most common among heavy drinkers in late middle age, and excessive alcohol consumption might impact ALDH2 activity.[43,44] In addition, individuals with other conditions such as liver disease or after gastro-intestinal surgery that might affect the breath ethanol or acetaldehyde levels were not tested in this study. Furthermore, a larger study in an independent validation cohort, including ADH1B*1/*1 allele carriers, heavy drinkers, and elderly persons, is required to confirm the applicability of this test as a screening tool.

In conclusion, our new breath test was demonstrated to be a useful tool for identifying carriers of the *ALDH2*2* allele, who are at high risk for ESCC and HNSCC. Quantitative measurement of breath A/E ratio might be an effective tool for assessment of individual risk of these cancers. We expect that knowing that they carry the *ALDH2*2* allele would contribute to the prevention of ESCC and HNSCC through their abstinence from alcohol and also to the early detection of these cancers through frequent endoscopic examination for habitual drinker.

CONFLICT OF INTEREST

Guarantor of the article: Manabu Muto, MD, PhD.
Specific author contributions: I.A. and M.M.: study concept and design. K.T. and M.H.: contribution to study design. Y.A., K.H., A.M. and M.F.: acquisition of questionnaire data. I.A., S.O., T.C. and M.M.: analysis and interpretation of data. I.A., S. O., T.C. and M.M.: drafting of the manuscript. I.A. and M.M.: statistical analysis. M.T., Y.N. and A.U.: administrative, technical, or material support. I.A., S.O. and M.M.: obtained funding. M.M.: study supervision.
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Potential competing interests: None declared.

Study highlights

WHAT IS CURRENT KNOWLEDGE

- ✓ Acetaldehyde, the first metabolite of ethanol, is a definite carcinogen for the esophagus, head, and neck.
- ✓ Carriers of the *ALDH2*2* allele are at high risk for head and neck or esophageal squamous cell carcinomas if they drink alcohol.
- ✓ There are no methods suitable for mass screening to identify *ALDH2*2* allele carriers.

WHAT IS NEW HERE

- ✓ We established a breath test that measures breath acetaldehyde and ethanol levels after ingestion of a very small amount of alcohol.
- ✓ *ALDH2*2* allele carriers can be identified by calculating the ratio of breath acetaldehyde-to-breath ethanol levels.
- ✓ In this study of 111 participants, the breath test could identify *ALDH2*2* allele carriers with 96.4% accuracy.

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