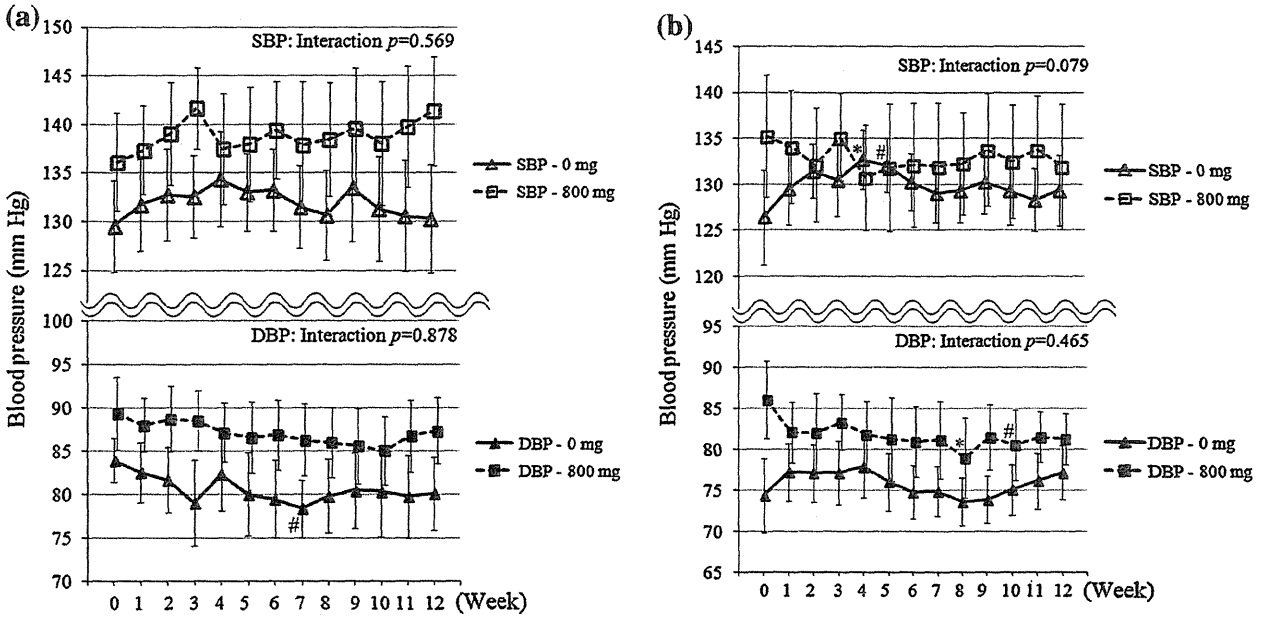

Fig. 3 Effect of *Umezu* polyphenols on blood pressure measured at home, **a** in the morning, and **b** at night. Time-dependent repeated-measure analyses and Dunnett's test were performed (vs. data in week 0). The interaction between intervention and time was also evaluated ($^{*}p < 0.05$; $^{\#}p < 0.10$). *SBP* systolic blood pressure, *DBP* diastolic blood pressure

Table 2 Effects of *Umezu* polyphenols on physical and mental component summaries in SF-8

	UP (mg/day)	Week 0		Week 4		Week 8		Week 12		Interaction *p*
		Mean	SD	Mean	SD	Mean	SD	Mean	SD	
PCS	0	52.19	5.13	52.08	3.78	51.28	6.13	49.07	5.29	0.512
	800	52.52	5.25	52.91	2.94	51.72	4.66	53.30	2.80	
MCS	0	41.46	9.88	44.79	8.67	43.88	4.49	42.71	9.20	0.231
	800	49.75	3.80	52.42	2.17	52.79	2.87	50.22	2.88	

Although time-dependent repeated-measure analyses and Dunnett's test were attempted (vs. data in week 0), analyses resulted in an error. Instead, summary scores in weeks 4, 8 and 12 were separately compared with the data in week 0 with paired *t* test, and none of them resulted in a significant outcome. Then, the interaction between intervention and time was evaluated

PCS physical component summary, *MCS* mental component summary, *UP Umezu* polyphenols

Safety and adherence

As for the safety of UP capsules, it was the winter season, when common cold was prevalent, and study subjects reported some signs and symptoms, mainly some respiratory and digestive ones. But these conditions were transient and recovered soon. And all the subjects competed the 12-week RCT. In SF-8, found were some nonsignificant improvements of the self-perceived physical condition in the polyphenol group, and some nonsignificant improvements of the self-perceived mental condition in both the groups (Table 2).

Discussion

In the current pilot study conducted in a double-blind randomized controlled trial (RCT), the beneficial effects of UP on hypertension were limited, probably due to the small number of study subjects. However, it showed the safety of UP capsules, even administered in a higher dose (800 mg daily) and for 12 weeks. Furthermore, the adherence of study subjects was good.

Wakayama Prefecture is higher in the mortality rate [20] and in the prevalence of cardiovascular diseases [21] compared with the average in Japan, which may be partly

due to the high prevalence in residents aged 65 years or older. On the other hand, several surveys reveal that residents in Wakayama have the highest [22] and the second highest (next to Shizuoka) [23] household expenditures on *Umeboshi* (pickled plums) and Mandarin oranges, respectively, and plum [13, 14] as well as citrus intake [24] are negatively associated with cardiovascular disease. Japanese plum is consumed mainly in the form of *Umeboshi*, which contains a considerable amount of sodium, or in the form of *Umeshu* (a Japanese liqueur made from steeping *Ume* fruits (while still unripe and green) in alcohol and sugar). Sodium in *Umeboshi* may elevate BP, while daily salt intake in Wakayama is lower than the average in Japan [25], and *Umeshu* does not give plum active ingredients to nondrinkers. In other words, the way in which Japanese plum is consumed may not contribute to substantial reduction of cardiovascular disease. Therefore, some more efficient ways to ingest plum active ingredients are desired. UP extracts, free of sodium and alcohol, can be provided in soft drinks [15] and in capsules, and they could serve as a solution to provide beneficial effects of Japanese plum.

Our previous study in 2011 [15] was as short as 5 weeks, and the maximum dose of UP was as low as 200 mg daily, which possibly led to the failure in showing significant and consistent findings regarding the BP-lowering effects of UP. To overcome these problems, a daily dose of 800 mg and a study period of 12 weeks were adopted in this study. Due to the length of the study, and the exclusion of those with hypertension grade 2 or greater as well as those under medication, the number of study subjects was limited. Besides, we encouraged workers with high-normal BP or hypertension without medication to join the study and exclude those with medication, maximizing the effects of UP.

The study population from which the study subjects derived is the personnel of the institute the principal investigator (ST) belongs to, and is likely to understand and cooperate to the study in general. The previous 5-week study in 2011 was also conducted thanks to this population [15]. However, the present study was as long as 12 weeks. In general, the longer the period of a study is, the less likely they are to participate in it. In addition, not a few hypertensive candidates were reportedly excluded before the study enrollment due to taking antihypertensive agents, which suggests that the personnel of the institute is aware of their own health. These conditions resulted in a small number of subjects. However, given the scarcity of RCTs of fruit polyphenols in humans evaluating for 12 weeks or longer, this study will provide another piece of valuable evidence in the study of polyphenols.

The safety of UP in experimental animals is already shown in a series of studies including a 90-day subchronic study in rats [16]. And this is the first study where UP were administered for 12 weeks in humans. Liu et al. [26] showed in a meta-analysis that the number of studies on fruit juice polyphenol with a study period of 12 weeks or 3 months is limited [27–29], and that there is only one study evaluating the effects of fruit polyphenols on BP as long as 3 months [27], which showed no significant outcomes. But in the overall meta-analysis, fruit juice reduced DBP significantly by 2.07 mmHg (95 % CI −3.75, −0.39 mmHg), but SBP insignificantly by 2.03 mm Hg (95 % CI −4.47, +0.41 mmHg). These results are partially consistent with findings of the present study. In another 12-week RCT among overweight Korean adults [30], a polyphenol extract from *Ecklonia cava* significantly contributed to lowering body fat and serum lipid parameters such as total and LDL cholesterols with dose dependence. It also had some BP-lowering effects, although its dose–response effect was not significant. Interventional studies of a longer period evaluating the effects of polyphenols among humans are very limited. In a 16-week study among patients under antihypertensive medication, BP reduction was observed in the extra-virgin olive oil group compared with the sunflower oil group. Enhanced nitric oxide levels stimulated by polyphenols might contribute to this beneficial effect [31].

As for SF-8, the physical condition improved in the polyphenol group and the mental condition improved in both the groups. In our previous study, the physical condition improved only in the polyphenol group and the mental condition improved only in the placebo group [15]. Given some placebo effects in the placebo group, these findings support some physically positive effects in UP. The biology of this improvement is yet to be clarified.

Several reasons may explain why the effects of UP against BP were limited. First, involvement of type 2 error should be taken into account. This RCT was a preliminary test, and it was conducted in a small number of subjects. This might have reduced the power to detect BP-lowering effects of UP. Second, some psychological factors might be involved in the decrease of BP in the placebo group, although it is unclear why this occurred. In our previous study [15], the MCS score significantly improved in the placebo group, but it did not in the UP group, which resulted in the diminished differences between UP and placebo groups.

Some study limitations should be mentioned. First, information on the subjects' age and sex was obtained during the recruitment of study participants and before the random allocation, but their BP was measured only after the random allocation and the beginning of the study, due to some logistic reasons. Therefore, baseline BP could not be utilized as the allocation variable. Second, it should be considered that study subjects ignored some "outliers" in BP since they were medical professionals; they might

reject some BP values that they subjectively considered extremely high or low as incorrect rather than recorded them "as is", and then measured BP repeatedly until they got values that they could regard as correct. These circumstances could throw some effects on the discrepancy in BP.

To overcome the above-mentioned limitations, we conducted a 14-week community-based RCT in 2013, where we randomly allocated a total of 72 study subjects into two arms, taking into account of their baseline BP as well as sex and age. Furthermore, to ensure objectivity in home BP self-measurement, we introduced a personal BP monitor which allowed home measurement data to be automatically transmitted to servers over 3G networks. We would like to report the results later.

In conclusion, the ability of UP to decrease BP is no more remarkable than that of a placebo. However, UP were shown safe and high in adherence, even administered in a higher dose (800 mg daily) and for a longer period (12 weeks).

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Conflict of interest The authors declare that they have no conflict of interest.

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Genetic Alcohol Sensitivity Regulated by *ALDH2* and *ADH1B* Polymorphisms as Indicator of Mental Disorders in Japanese Employees

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Abstract — **Aims:** Alcohol-related disorders (ARD) have been shown to be accompanied by a variety of other comorbid mental disorders. This study evaluated the associations between a variety of mental disorders and genetic alcohol sensitivity. **Methods:** A total of 1944 Japanese workers were interviewed regarding their mental disorders by the Mini-International Neuropsychiatric Interview (M.I.N.I.). We investigated the relationship of *ADH1B* rs1229984 and *ALDH2* rs671 polymorphisms' combination with mental disorder risks. Logistic regression analysis was used to evaluate the associations between those polymorphisms and mental disorders, adjusting for sex, age, and job rank. **Results:** The degree of alcohol sensitivity was classified into five groups according to the combination of *ADH1B* and *ALDH2* genotypes (Group I–V in order starting from the lowest alcohol sensitivity). Those with *ALDH2 *1/*1* and *ADH1B *1/*1* or with *ALDH2 *1/*1* and *ADH1B *1/*2,*2/*2* (low sensitivity) were significantly or nearly significantly associated with an increased risk of ARD compared with those with *ALDH2 *1/*2* and *ADH1B *1/*2,*2/*2* as a reference. Those with *ALDH2 *1/*1* and *ADH1B *1/*1* were also likely to be at an increased risk of any mental disorder except ARD, as well as disorders without comorbid ARD. This tendency was more apparent among women (OR 11.94, 95% CI 0.73–195.63) and non-drinkers (OR 5.43, 95% CI 1.05–28.23). **Conclusion:** The genotype combination of *ALDH2 *1/*1* and *ADH1B *1/*1* is significantly associated with an increased risk of any mental disorder, especially ARD. Non-drinkers or women with *ALDH2 *1/*1* and *ADH1B *1/*1* are likely to suffer from any mental disorder except ARD.

INTRODUCTION

Alcohol-related disorders (alcohol dependence/abuse, ARD) have been shown to be a critical cause of depression or suicide (Schneider, 2009; Beghi *et al.*, 2013). Given that ARDs are accompanied by a variety of comorbid psychiatric disorders such as bipolar, anxiety, eating and psychotic disorders (Suzuki *et al.*, 2011; Farren *et al.*, 2012; Lev-Ran *et al.*, 2012; Charriau *et al.*, 2013), and that ARD are among the most prevalent psychiatric disorders (Lev-Ran *et al.*, 2012), alcohol sensitivity may affect not only ARD but also a broad range of psychiatric disorders. Though ARD occurs on the basis of psychological vulnerability, moderate alcohol consumption has been shown to be effective for psychological stress reduction (Peele and Brodsky, 2000; Marchard *et al.*, 2003). Since alcohol consumption generally increases in accordance with increased mental strain, a part of moderate drinkers is considered to be affected with ARD (Keyes *et al.*, 2012).

Thus, heavier drinkers (i.e. those with low alcohol sensitivity) tend to suffer from many alcohol-related problems stemming from ARD, leading to isolation from social support and suffering from mental illness. On the other hand, it has been suggested that drinking might help people cope with mental stress (Eckardt *et al.*, 1998; Moore *et al.*, 2007; Anthenelli, 2012; Watt *et al.*, 2014). However, this method may not work for people who cannot drink because of their high alcohol sensitivity, which, in turn, leads to accumulation of the stress. Thus, there is a possibility that genetic alcohol sensitivity is associated with a broad range of mental disorder risks.

Alcohol metabolism occurs in two major steps: oxidation of alcohol to acetaldehyde by the alcohol dehydrogenase enzymes, and further oxidation of acetaldehyde into acetate by aldehyde dehydrogenase enzymes, ALDH2. Single nucleotide polymorphisms (SNPs) of the two enzymes' gene loci, *ADH1B* rs1229984 and *ALDH2* rs671 SNPs, which show different alcohol/acetaldehyde oxidizing capabilities among individuals, have been reported to exert significant impacts on alcohol consumption and on the risk for ARD in East Asia populations (Higuchi *et al.*, 1996; Takeshita *et al.*, 1996; Whitefield, 2002; Kim *et al.*, 2008).

The *ADH1B*2* allele represents a much higher activity of ADH1B than the homozygotes for the *ADH1B*1* form, enabling fast alcohol elimination and acetaldehyde accumulation in the blood after drinking (Bosron and Li, 1986; Yoshida *et al.*, 1991), increasing alcohol sensitivity, and as a result, reducing the risk for ARD. The *ALDH2*2* allele encodes a catalytically inactive subunit (Bosron and Li, 1986; Yoshida *et al.*, 1991), which causes alcohol-related adverse physical reactions such as flushing, palpitation, nausea, headache and general discomfort (Matsuo *et al.*, 2006). These adverse reactions in subjects with *ALDH2*2*, which are due to excessive acetaldehyde accumulation, tend to increase alcohol sensitivity and inhibit drinking, subsequently playing a protective role against ARD.

Thus, both mutant alleles of *ADH1B*2* and *ALDH2*2* are considered to be protective against ARD, while wild alleles of *ADH1B*1* and *ALDH2*1* have been shown to promote ARD among drinkers (Kim *et al.*, 2008). More concretely, those with the combination of wild homozygote alleles of the two enzymes' gene loci, *ALDH2*1/*1* and *ADH1B*1/*1*, are expected to be at the greatest risk for ARD. On the other hand, those who have the combination of mutant homozygote alleles of both loci, *ALDH2*2/*2* and *ADH1B*2/*2*, are considered to be least likely to contract ARD. For the sake of clinical convenience, degrees of alcohol sensitivity regulated by the two enzymes' gene loci can be classified into the following five groups in order of lowest alcohol sensitivity: Group I (*ALDH2 *1/*1* and *ADH1B *1/*1*), Group II (*ALDH2 *1/*1* and *ADH1B *1/*2,*2/*2*), Group III (*ALDH2 *1/*2* and *ADH1B*

**1/*1*), Group IV (*ALDH2 *1/*2* and *ADH1B *1/*2,*2/*2*) and Group V (*ALDH2 *2/*2* and *ADH1B *1/*1, *1/*2,*2/*2*) (Yang *et al.*, 2010; Yokoyama *et al.*, 2010).

Although a few studies have been conducted to determine the combined genetic effect of *ALDH2* and *ADH1B* on ARD (Kim *et al.*, 2008; Yao *et al.*, 2011), there is almost no evidence regarding the combined effect of these two loci on other mental disorders (Hishimoto *et al.*, 2010). The purpose of the current study is to investigate whether subjects with high alcohol sensitivity have low rates of ARD in the Japanese working population, and to clarify the associations between genetic alcohol sensitivity regulated by *ALDH2* and *ADH1B* and a broad range of mental disorders, with explicit assessment of the effects of comorbid ARD as well as gender differences and drinking habit.

METHOD

Participants

Our subjects were 2442 local government employees in the Kinki area of Japan who underwent annual health checkups from May to July 2013. Their work included a variety of clerical jobs, along with jobs in monitoring, security and communication service. The investigators encouraged all employees to enroll in the study; 1944 of whom (79.6%) agreed to participate in an interview survey regarding mental disorders, and provided blood samples to determine the two enzyme genetic polymorphisms. All participants gave written consent. This study was approved by the institutional review board for genetic research of Wakayama Medical University (acceptance number 106).

Psychiatric Structured Interview

The Mini-International Neuropsychiatric Interview (M.I.N.I.), Japanese version 5.0.0 (2003) (Sheehan *et al.*, 1998; Sheehan and Lecrubier, 2003), a conveniently structured tool designed to identify cases of mental disorder, was used for the present interview survey. The reliability and validity of the Japanese version of the M.I.N.I. were reported to be satisfactory (Otsubo *et al.*, 2005), by comparison between M.I.N.I. and Structured Clinical Interview for DSM-III-R as well as by interrater and test-retest reliabilities. A total of 14 interviewers, all of whom were licensed doctors or nurses, were enrolled as competent to conduct the interviews. The first author (K.Y.), a psychiatrist, trained them in essential interview skills, including didactic sessions of the general interview, and reviews of the instrument sections. Furthermore, the first author checked the interviewers and corrected them as the need arose during interview sessions so that the interview could be conducted appropriately.

The M.I.N.I. deals with 17 Axis I mental disorders based on the standard of a 12-month prevalence of 0.5% or more (Sheehan *et al.*, 1998), among which we checked the disorders listed in the first screening session: major depressive disorder, dysthymia, suicidality, manic episode, panic disorder, agoraphobia, social phobia, specific phobia, obsessive-compulsive disorder, general anxiety disorder, ARD, substance dependence/abuse, anorexia nervosa, anorexia bulimia and post-traumatic stress disorders (PTSD), were classified as candidate disorders potentially associated with genetic alcohol sensitivity. These disorders are all included in the M.I.N.I., except psychotic disorders and anti-social personality disorder. All subjects were asked the screening questions essential for the diagnosis of these disorders. If their mental symptoms satisfied those questions, more detailed questions were used to arrive at a final diagnosis of each disorder.

Genetic analysis

Genomic DNA was extracted from blood of 1296 samples using Nucleo Spin Blood Kit (Takara Bio, Otsu). Genetic determinations were made by examiners while blinded to the mental disorder status. TaqMan® SNP genotyping assays purchased from Applied Biosystems (Foster City, CA, USA) were used for the following (gene, SNP, assay ID): *ADH1B*, rs1229984, C_2688467_20; *ALDH2*, rs671, C_11703892_10. The remaining 648 samples were directly genotyped by the TaqMan assay on an ABI 7300 Real Time PCR System (Hayashida *et al.*, 2014).

Statistical analysis

The *P*-value for Hardy–Weinberg equilibrium (HWE) was calculated as the difference between the number of genotypes and the number of alleles (df = 1). As mentioned above, the interview procedure of M.I.N.I. consists of two steps, i.e. a screening step for any mental disorders, and a detailed interview for each disorder found in the screening step. If the subjects answered 'yes' to any screening questionnaire, they were regarded as having a 'preliminary' diagnosis. Final diagnoses were given if the subjects met the final diagnostic criteria of each disorder. Four models were created for examining the associations between mental disorders and the two enzyme genetic polymorphisms based on the definition of outcome variables (Fig. 1). These outcomes were: (a) alcohol-related disorders, (b) any mental disorders except alcohol-related disorders, (c) any mental disorders *without* comorbid alcohol-related disorders and (d) any preliminary and final diagnoses of mental disorders. Those who did not correspond to any screening questions except ARD were categorized as normal controls ($n = 1437$). The screening question for ARD, 'In the past 12 months, have you had three or more alcoholic drinks within a three-hour period on three or more occasions?', was used as a proxy for a drinking habit. 'Three or more alcoholic drinks' in the Japanese version means three or more glasses (three or more units on average) of any alcoholic beverage. The Japanese 'standard drink' of alcoholic is 25 ml, which is 2.5 times that of the UK standard. A detailed interview was conducted on those who answered 'yes' to this screening question (i.e. drinkers) to confirm ARD defined by DSM-IV and ICD-10. The detailed interview consisted of seven questions for alcohol dependence and four questions for alcohol abuse. Alcohol abuse was confirmed when the subjects did not meet the criteria for alcohol dependence.

Logistic regression analysis was used to obtain odds ratios (ORs) and 95% confidence intervals (CIs). The dependent variables in that analysis were the four outcomes defined above, which were compared with the 1437 controls. Explanatory variables were *ALDH2* and *ADH1B* genotypes classified as mentioned in the Introduction (Group I–V), adjusting for age, sex and job rank. Age was divided into two categories, <40 or 40+, since two peaks of age distribution were observed at the dividing line of 40 years of age. Job rank was also divided into two categories, administrative position or not.

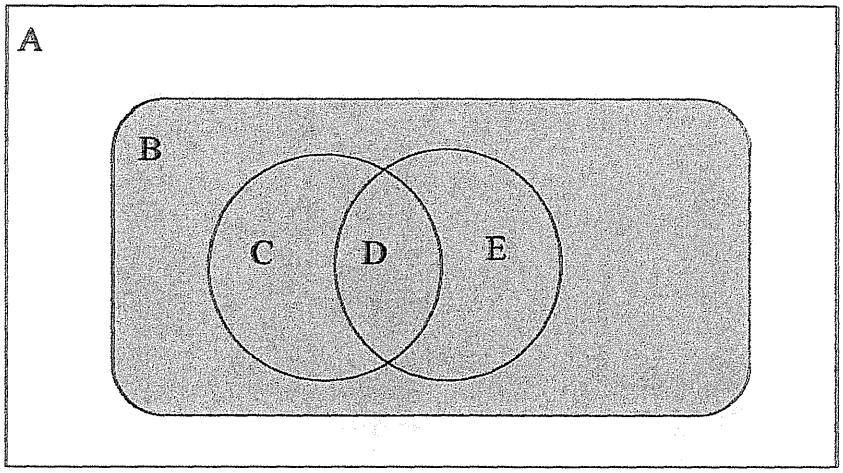

Fig. 1. Definition of outcome variables (Models 1–4). (A) Controls ($n = 1437$). (B) Any preliminary and final diagnoses ($n = 507$, Model 4). (C+D) Alcohol-related disorders ($n = 168$, Model 1). (D+E) Any mental disorders except alcohol-related disorders ($n = 68$, Model 2). (E) Any mental disorders *without* comorbid alcohol-related disorders ($n = 57$, Model 3). (B) includes (C) ($n = 157$), (D) ($n = 11$) and (E) ($n = 57$). (A) ($n = 1437$) + (B) ($n = 507$) = entire sample ($n = 1944$).

The ORs and their 95% CIs were obtained from the corresponding logistic regression coefficients and their standard errors. Each OR showed how many times subjects with the genotypes were likely to have been affected by mental disorders compared with Group IV. Group IV was set as the reference group since its members were considered better able to control their alcohol-related behaviors compared with the other lower sensitivity groups (Yokoyama *et al.*, 2010). These multivariate analyses were conducted separately for drinkers and non-drinkers, as well as the entire sample. We also estimated trends of the OR in each model in accordance with the number of wild homozygotes in the two loci. Gene-gene interaction was evaluated by likelihood ratio test in the entire sample with males and females combined. *P*-values (two-sided) <0.05 were considered statistically significant. All computations were performed using the SAS software package, version 9.1.3 (SAS Institute, Inc., Cary, NC, USA).

RESULTS

Distributions of mental disorders as well as demographic backgrounds of the subjects are shown in Table 1. Nearly 90% of the subjects were male and a little more than 10% were employed at administrative positions. The prevalence of mental disorders was low since the subjects were generally from a healthy working population. However, 26.1% of them had some signs of mental disorders. The most frequent disorders were ARD (8.6%). There were 68 subjects who suffered from mental disorders except ARD, while 11 of them had ARD. Thus, only 11 subjects (0.6%) had ARD as well as other comorbid psychiatric conditions, and 57 (2.9%) had mental disorders other than ARD alone. Assuming that the proportion of those with Group II was 50% based on our previous survey (Takeshita, 2012), and that the expected OR of any mental disorder except ARD associated with this group was 3.0 with a statistical power of 0.80, the required sample size of affected cases was calculated to be 66, almost equal to the current results.

Table 1. Demographic background and frequencies of mental disorders in study subjects ($n = 1944$)

Variables	*n* (%)
Male	1742 (89.6)
Administrative position	249 (12.8)
Age (mean (SD))	37.9 (11.6)
Mental disorders	
Major depressive disorder	20 (1.0)
Dysthymia	4 (0.2)
Suicidality[a]	13 (0.7)
Hypomanic or manic episode[b]	18 (0.9)
Panic disorder	1 (0.05)
Agoraphobia	4 (0.2)
Specific phobia	2 (0.1)
Obsessive-compulsive disorder	8 (0.4)
Post-traumatic stress disorder	0 (0.0)
Alcohol-related disorders[c,d]	168 (8.6)
Other substance-related disorders	2 (0.1)
Eating disorders[e]	7 (0.4)
General anxiety disorders	12 (0.6)
Any mental disorders except alcohol-related disorders[d]	68 (3.5)
Any mental disorders without comorbid alcohol-related disorders[d]	57 (2.9)
Any mental disorders including preliminary and final diagnosis[d]	507 (26.1)

[a]Including one with lifetime history of suicide attempt. [b]Current and past episode. [c]Alcohol dependence and abuse. [d]Used as outcome variables in the multiple logistic regression analyses. [e]Anorexia and bulimia nervosa.

The distributions of *ALDH2* and *ADH1B* polymorphisms as well as their combined classification (Group I–Group V) separately for men and women as well as entire samples are shown in Table 2. Distributions between men and women were quite similar, and no significant deviation was detected by HWE among the subjects for both *ALDH2* and *ADH1B* in men and women as well as whole sample. Group II was, as expected, most frequent, and the next most frequent group was Group IV. Group III had the fewest members among the five groups. These genotype distributions were consistent with a previous Japanese study (Takeshita, 2012).

Table 2. Frequencies of *ALDH2* and *ADH1B* polymorphisms and genotype classification according to the polymorphisms in study subjects

	Men, *n* (%) (*n* = 1742)	Women, *n* (%) (*n* = 202)	Total, *n* (%) (*n* = 1944)
ALDH2			
**1/*1*	875 (50.2)	111 (55.0)	986 (50.7)
**1/*2*	700 (40.2)	76 (37.6)	776 (39.9)
**2/*2*	167 (9.6)	15 (7.4)	182 (9.4)
HWE *P*-value	0.12	0.70	0.10
ADH1B			
**1/*1*	89 (5.1)	8 (4.0)	97 (4.9)
**1/*2*	630 (36.2)	74 (36.6)	704 (36.2)
**2/*2*	1023 (58.7)	120 (59.4)	1143 (58.8)
HWE *P*-value	0.53	0.41	0.39
Genotype classification			
Group I (*ALDH2 *1/*1* and *ADH1B *1/*1*)	39 (2.2)	6 (3.0)	45 (2.3)
Group II (*ALDH2 *1/*1* and *ADH1B *1/*2,*2/*2*)	836 (48.0)	105 (52.0)	941 (48.4)
Group III (*ALDH2 *1/*2* and *ADH1B *1/*1*)	41 (2.4)	2 (1.0)	43 (2.2)
Group IV (*ALDH2 *1/*2* and *ADH1B *1/*2,*2/*2*)	659 (37.8)	74 (36.6)	733 (37.7)
Group V (*ALDH2 *2/*2* and *ADH1B *1/*1, *1/*2,*2/*2*)	167 (9.6)	15 (7.4)	182 (9.4)

HWE, Hardy–Weinberg equilibrium.

Table 3 shows the association between *ALDH2* as well as *ADH1B* polymorphisms according to their classification, and mental disorders separately for men and women. Group I and Group II were marginally significantly or significantly associated with an increased risk of ARD in men. Group I of women shows >25-fold increased risk of ARD while the 95% CI was too wide due to the small sample size. Group V was significantly associated with less than one-tenth of the decreased prevalence of ARD compared with Group IV in men. Group II showed a modest but significant increase in the risk for any mental disorder (including preliminary diagnoses) in men.

The association between the Group I–V polymorphism classification and mental disorders was evaluated separately for drinkers and non-drinkers (Table 4). Although Group I showed non-significant, modest associations with an increased risk of mental disorders defined as the four outcomes among drinkers, the association between Group I and any mental disorders other than ARD was strong (>5-fold increased risk) and significant in non-drinkers. In this analysis of non-drinkers, 'any mental disorders except alcohol-related disorders' (model 2) and 'any mental disorders *without* comorbid alcohol-related disorders' (model 3) had the same outcomes, yielding the same results.

Trends in associations between mental disorders and the two enzyme polymorphisms according to the number of the high-risk genotypes in the two loci (0, 1, and 2) are shown in Table 5. Significant trends were observed for ARD and any mental disorders including preliminary diagnoses, whereas trends for 'any mental disorders except alcohol-related disorders' (model 2) and 'any mental disorders *without* comorbid alcohol-related disorders' (model 3) were far from statistically significant. When those analyses were conducted separately for drinkers and non-drinkers, calculated statistics were far from significant (data not shown), except for a non-significant modest trend for any mental disorders including preliminary diagnosis ($P = 0.096$) among drinkers. Gene-gene interactions between *ALDH2* and *ADH1B* on mental disorders were not statistically significant ($P = 0.36$ for 'any mental disorders except alcohol-related disorders', 0.32 for 'any mental disorders *without* comorbid alcohol-related disorders', 0.37 for any mental disorders including preliminary diagnoses, and 0.16 for ARD).

DISCUSSION

Individual alcohol sensitivity is strongly regulated by the combination of *ALDH2* and *ADH1B* genotypes. The current study first applied the classification of the combined effects of the two alcohol metabolic enzymes' polymorphisms (i.e. Group I-V) to the prevalence of various kinds of mental disorders. Compared with Group IV (*ALDH2 *1/*2* and *ADH1B *1/*2, *2/*2*), who are considered to have self-inhibition against alcohol-related behaviors, Group I (*ALDH2 *1/*1* and *ADH1B *1/*1*) and Group II (*ALDH2 *1/*1* and *ADH1B *1/*2,*2/*2*) were at a significantly elevated risk for ARD, especially in women. The genotype combination of Group I also tended to be associated with an increased risk of other mental disorders, as well as such disorders *without* comorbid ARD, while there were no material associations between Group II and those disorders. Such associations of Group I and mental disorders except or without ARD were more apparent in women and non-drinkers. In addition, many of the high-risk genotypes in the two loci (alleles related to low alcohol sensitivity) were significantly associated with ARD and any mental disorders, including preliminary diagnoses.

These findings suggest that those with low alcohol sensitivity are more likely to be affected with ARD than those with high alcohol sensitivity, consistent with previous findings (Kim *et al.*, 2008; Yokoyama *et al.*, 2010; Yao *et al.*, 2011). The observed strong associations between Group I and ARD as well as other mental disorders in women should be interpreted with caution because few women had such mental disorders, leading to non-significant findings. Female alcohol-dependents have been shown to be more likely to have psychiatric comorbidities than male alcohol-dependents (Higuchi *et al.*, 1993; Grant and Harford, 1995; Kessler *et al.*, 1997), consistent with the current findings. On the other hand, effects of *ALDH2* and *ADH1B* were reported to differ between men and women; female alcohol-dependents with *ALDH2*2* were shown to develop ARD earlier than those with *ALDH2*1*,

Table 3. Associations between *ALDH2* and *ADH1B* polymorphisms and mental disorders in men and women

	Model 1[a]		Model 2[b]		Model 3[c]		Model 4[d]	
Groups	OR	95% CI	OR	95% CI	OR	95% CI	OR	95% CI
Men								
($n = 1742$)								
Group I	2.51	0.91–6.92	2.34	0.66–8.31	2.51	0.70–8.96	1.46	0.72–2.96
Group II	2.27	1.55–3.32	0.88	0.50–1.55	0.73	0.39–1.37	1.32	1.05–1.67
Group III	2.43	0.88–6.67	0.73	0.10–5.58	0.81	0.11–6.23	1.52	0.77–3.00
Group IV	1.00	ref	1.00	ref	1.00	ref	1.00	ref
Group V	0.09	0.01–0.69	0.46	0.14–1.56	0.51	0.15–1.74	0.80	0.52–1.22
Women								
($n = 202$)								
Group I	26.29	1.14–608.06	7.86	0.54–114.33	11.94	0.73–195.63	4.08	0.71–23.34
Group II	3.18	0.35–29.28	1.34	0.31–5.85	2.00	0.37–10.72	1.42	0.70–2.86
Group III	NA		NA		NA		NA	
Group IV	1.00	ref	1.00	ref	1.00	ref	1.00	ref
Group V	NA		1.63	0.15–17.23	2.42	0.20–29.19	0.56	0.12–2.76

Adjusted for age and job rank in each model. Controls are 1285 for men and 152 for women without any preliminary or final psychiatric diagnoses in all four models. NA = not available due to lack of subjects within the categories. Group I (*ALDH2 *1/*1* and *ADH1B *1/*1*); Group II (*ALDH2 *1/*1* and *ADH1B *1/*2, *2/*2*); Group III (*ALDH2 *1/*2* and *ADH1B *1/*1*); Group IV (*ALDH2 *1/*2* and *ADH1B *1/*2,*2/*2*); Group V (*ALDH2 *2/*2* and *ADH1B *1/*1, *1/*2,*2/*2*). [a]Alcohol-related disorders, $n = 1447$ for men and 158 for women. [b]Any mental disorders except alcohol-related disorders, $n = 1343$ for men and 162 for women. [c]Any mental disorders *without* comorbid alcohol-related disorders, $n = 1333$ for men and 161 for women. [d]Any mental disorders including preliminary and final diagnosis, $n = 1742$ for men and 202 for women.

Table 4. Associations between *ALDH2* and *ADH1B* polymorphisms and mental disorders among drinkers and non-drinkers

	Model 1[a]		Model 2[b]		Model 3[c]		Model 4[d]	
	OR	95% CI	OR	95% CI	OR	95% CI	OR	95% CI
Drinkers[e]								
($n = 1149$)								
Groups								
Group I	1.95	0.74–5.16	1.68	0.35–7.96	2.09	0.43–10.23	1.33	0.62–2.85
Group II	1.65	1.12–2.43	0.92	0.47–1.79	0.87	0.40–1.90	1.26	0.96–1.66
Group III	1.87	0.66–5.34	NA		NA		1.39	0.63–3.10
Group IV	1.00	ref	1.00	ref	1.00	ref	1.00	ref
Group V	0.45	0.06–3.51	NA		NA		0.54	0.15–1.89
Non-drinkers[e]								
($n = 795$)								
Groups								
Group I	–	–	5.43	1.05–28.23	5.43	1.05–28.23	2.12	0.62–7.27
Group II	–	–	0.74	0.29–1.89	0.74	0.29–1.89	1.02	0.67–1.54
Group III	–	–	1.98	0.24–16.60	1.98	0.24–16.60	1.15	0.31–4.24
Group IV	–	–	1.00	ref	1.00	ref	1.00	ref
Group V	–	–	0.67	0.21–2.09	0.67	0.21–2.09	1.06	0.66–1.70

Group I (*ALDH2 *1/*1* and *ADH1B *1/*1*); Group II (*ALDH2 *1/*1* and *ADH1B *1/*2,*2/*2*); Group III (*ALDH2 *1/*2* and *ADH1B *1/*1*); Group IV (*ALDH2 *1/*2* and *ADH1B *1/*2,*2/*2*); Group V (*ALDH2 *2/*2* and *ADH1B *1/*1, *1/*2,*2/*2*). [a]Alcohol-related disorders, $n = 964$. [b]Any mental disorders except alcohol-related disorders, $n = 837$ for drinkers and 668 for non-drinkers. [c]Any mental disorders without comorbid alcohol-related disorders, $n = 826$ for drinkers and 668 for non-drinkers. [d]Any mental disorders including preliminary and final diagnosis, $n = 1149$ for drinkers and 795 for non-drinkers. [e]Those who had alcoholic drinks within a 3-h period on three or more occasions in the past year (drinkers) or did not (non-drinkers). Adjusted for age, sex and job rank in each model. NA, not available due to lack of subjects within the categories.

resulting in having other psychiatric comorbidities (Kimura *et al.*, 2011). Since subjects of this study were hospitalized patients, it might be difficult to apply their results to the general working population.

The strong associations between Group I or Group II and ARD may be among the causes that explain the positive associations between those genotype combinations and overall mental disorders (model 4). On the other hand, those with *ALDH2 *1/*1* and *ADH1B *1/*1* were at an elevated risk for mental disorders except or without ARD, while Group II and Group III were not. Unexpectedly, this tendency was more apparent among women or those without actual drinking habits. Non-drinkers in Group I were confronted by a more than 5-fold increased risk of mental disorders except ARD, compared with those of Group IV.

Thus, although those with low alcohol sensitivity tended to be affected with ARD, their other mental problems, especially for those with very low sensitivity (Group I), seem not to come directly from ARD or its related behavioral and psychosocial adversities. The reason why Group I was strongly associated with mental disorders excluding ARD among non-drinkers is unclear. One possible explanation is that it is beneficial to drink alcohol for those with very low alcohol sensitivity to alleviate undesired emotional states. Such behavioral effects of moderate

Table 5. Associations between number of **1/*1* in the two loci and mental disorders in subjects except Group V

	Model 1[a]		Model 2[b]		Model 3[c]		Model 4[d]	
Groups (no. of **1/*1*)	OR	95% CI	OR	95% CI	OR	95% CI	OR	95% CI
Group I (2)	2.97	1.16–7.62	2.73	0.89–8.31	3.06	0.99–9.41	1.66	0.87–3.16
Group II + III (1)	2.29	1.58–3.32	0.91	0.54–1.53	0.83	0.47–1.47	1.33	1.07–1.66
Group IV (0)	1.00	ref	1.00	ref	1.00	ref	1.00	ref
P for trend	<0.0001		0.60		0.67		0.006	

Adjusted for age, sex and job rank in each model. Controls are 1290 subjects without any preliminary or final psychiatric diagnoses in all four models. Group I (*ALDH2 *1/*1* and *ADH1B *1/*1*); Group II (*ALDH2 *1/*1* and *ADH1B *1/*2,*2/*2*); Group III (*ALDH2 *1/*2* and *ADH1B *1/*1*); Group IV (*ALDH2 *1/*2* and *ADH1B *1/*2,*2/*2*); Group V (*ALDH2 *2/*2* and *ADH1B *1/*1, *1/*2,*2/*2*). [a]Alcohol-related disorders, $n = 1457$. [b]Any mental disorders except alcohol-related disorders, $n = 1354$. [c]Any mental disorders *without* comorbid alcohol-related disorders, $n = 1343$. [d]Any mental disorders including preliminary and final diagnosis, $n = 1762$.

drinking can encompass events that the human or other animal can perceive as reinforcing through either positive (e.g. pleasurable) or negative (e.g. stress reduction) reinforcement mechanism (Eckardt *et al.*, 1998). However, if they cannot drink for any reason, the accumulated stress is expected to be serious enough to lead them to many kinds of mental disorders.

Another explanation is that Group I members are few among the Japanese, while a majority of the European population (Caucasians) are included in this group (Lorenzo *et al.*, 2006; Kayaalti and Söylemezoğlu, 2010). In regions where Group I is popular, where drinking habits are considered relatively uniform, those of Group I can drink heavily without hesitating. However, in Japan, it might be difficult for them to drink as much as they want, given their surroundings. As a reaction to such stress, they might quit drinking and then develop some mental disorders.

Third, impulsivity-related personality traits such as lack of premeditation, lack of perseverance, sensation seeking, negative urgency, positive urgency and reward sensitivity have been generally shown to be associated with alcohol consumption and problematic alcohol use (Stautz and Cooper, 2013). Thus, some specific personality traits linked with Group I may lead to a variety of mental disorders.

Strengths and limitations

The results in this study should be interpreted in the context of their strengths and limitations. Two important methodological strengths are noteworthy. First, this is the first study to investigate the relationships between various kinds of mental disorders with *ALDH2* and *ADH1B* polymorphisms, unlike the majority of previous studies on this issue which focused on ARD only. Second, we studied a large sample of employees who allowed us to conduct statistical analyses in accordance with the combination of the two enzymes' genotypes (i.e. Group I–V classifications).

On the other hand, three potential limitations are noteworthy. In spite of the large sample size, few subjects were having mental disorders except or without ARD evaluated by the structured interview since our subjects were generally healthy employees, and not those recruited from patients having mental disorders. Although a few of the subjects might have been found to have disorders partially because of the M.I.N.I. that evaluates the point (current) prevalence rather than the 12 months or lifetime prevalence, severity of the mental disorders is not considered to be so grave as to hinder working, which, in turn, is not always representative of the clinical disorders. ARD are also considered not so severe; rather, it might even be somewhat overestimated due to the drinking culture specific to the male-dominant workplace, leading to difficulty in generalizing the findings. However, prevalence of ARD was considered to be relatively accurate compared with the other disorders since it was estimated at 12 months prevalence.

Next, the reason why subjects with *ALDH2 *1/*1* and *ADH1B *1/*1* among the non-drinkers did not drink was unclear. Several possible interpretations for why they suffered from mental disorders except ARD depended on whether they wanted to drink or not. For those who wanted to drink, some mental disorders arose from the psychological frustration of desiring drinking, as mentioned above. However, if they disliked drinking, the mechanism that explains the associations between Group I and mental disorders except ARD would be difficult to understand and would remain inconclusive. One speculation is that some personality traits leading to mental disorders might be genetically linked with the genotype combination of Group I.

Lastly, as the onset age of mental disorders could not be confirmed by M.I.N.I., it could not be ascertained whether or not ARD preceded the other comorbid mental disorders. In other words, one cannot determine which mental disorder is alcohol-induced or independent. However, it is noteworthy that only 6.5% of the subjects with ARD had comorbid mental disorders.

CONCLUSIONS

The current study demonstrates that alcohol sensitivity regulated by *ALDH2* and *ADH1B* polymorphisms may be a useful indicator of mental disorders. Further larger scale, cross-cultural, clinical or population-based studies undoubtedly will lead to a more thorough understanding of the role of gene polymorphisms related to alcohol metabolism in the development of mental illness.

AUTHORS' CONTRIBUTIONS

K.Y. is principle investigator and wrote the draft of the manuscript. K.Mu., M.Has., M.Hay. and K.K. conducted genetic analysis. S.T. and K.T. assisted the field survey. T.T. gave the principle investigator special advices in interpreting the findings. K.Mi. superintended the entire study.

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Genetic alcohol sensitivity regulated by *ALDH2* and *ADH1B* polymorphisms is strongly associated with depression and anxiety in Japanese employees

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ABSTRACT

Background: Although alcohol-related disorders (ARD) have been shown to be accompanied by comorbid depressive and anxiety disorders, and alcohol metabolic enzyme genes, *ADH1B* and *ALDH2* polymorphisms, have been associated with an increased risk of ARD, no studies have been conducted to evaluate the associations between these genetic polymorphisms and anxiety or depression.
Method: A total of 1944 Japanese workers were interviewed regarding their depressive and anxiety disorders, including suicidality, by a brief psychiatric structured interview (MINI). We investigated the relationship of *ADH1B* rs1229984 and *ALDH2* rs671 polymorphism combinations with mental disorder risks. Logistic regression analysis was used to evaluate the associations between those polymorphisms and anxiety/depressive disorders, adjusting for sex, age, and job rank. The degree of alcohol sensitivity was classified into five groups according to the combination of two enzyme genotypes (Group I–V, in order from the lowest alcohol sensitivity).
Results: Those with *ALDH2* *1/*1 and *ADH1B* *1/*1 were likely to be at an increased risk of depressive and anxiety disorders as well as ARD. This tendency was more apparent among non-drinkers (OR 9.20, 95% CI 1.66–50.89). No adverse effects of *ALDH2* or *ADH1B* alone were observed with mental disorder risks. Likewise, analyses conducted combining job rank and genetic alcohol sensitivity showed no material associations with such risks.
Conclusions: Genetic alcohol sensitivity, especially that with the genotype combination of *ALDH2* *1/*1 and *ADH1B* *1/*1, was significantly associated with an increased risk of depressive and anxiety disorders as well as ARD.

1. Introduction

Alcohol-related disorders (alcohol dependence/abuse, ARD) have been shown to be among the critical causes of suicide (Beghi et al., 2013; Schneider, 2009; Yoshimasu et al., 2008). Abundant evidence has suggested that ARD is also associated with other comorbid psychiatric disorders, especially internalizing disorders such as anxiety or depression (Baker et al., 2012; Charriau et al., 2013; Saraceno et al., 2009). Two contrasting pathways hypothetically explain ARD and depression or anxiety disorders. One environmental pathway is that hard drinkers (i.e., those with low alcohol sensitivity) are likely to have any alcohol-related problem that can stem from ARD, leading to isolation from society and suffering from depression and anxiety, in addition to the direct depressogenic effect of ethanol (Abraham and Fava, 1999). Another hypothesis is that those who have a "poor head for drink" (i.e., those with high alcohol sensitivity) cannot relieve their mental stress by moderate drinking, as moderate alcohol consumption has been shown to be effective for psychological stress reduction (Marchard et al., 2003; Peele and Brodsky, 2000). Consequently, they

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accumulate mental strain as well as an inadequate drinking habit (i.e., ARD), leading them to a psychologically unstable state. In the former case, problematic alcohol use precedes internalizing symptomatology while the latter case is the opposite, thus remaining a matter of controversy (Saraceno et al., 2009).

Alcohol metabolism occurs in two major steps: oxidation of alcohol to acetaldehyde by the alcohol dehydrogenase enzymes, and further oxidation of acetaldehyde into acetate by aldehyde dehydrogenase enzymes, ALDH2. Single nucleotide polymorphisms (SNPs) of the two enzymes' gene loci, *ADH1B* rs1229984 and *ALDH2* rs671 SNPs, which show different alcohol/acetaldehyde oxidizing capabilities among individuals, have been reported to exert significant impacts on alcohol consumption and on the risk for ARD in East Asia populations (Higuchi et al., 1996; Kim et al., 2008; Takeshita et al., 1996; Whitefield, 2002).

The *ADH1B*2* allele represents a much higher ADH1B activity than the homozygotes for the *ADH1B*1* form, enabling fast alcohol elimination and acetaldehyde accumulation in the blood after drinking (Bosron and Li, 1986; Yoshida et al., 1991), increasing alcohol sensitivity, and as a result, reducing the risk for ARD. The *ALDH2*2* allele encodes a catalytically inactive subunit (Bosron and Li, 1986; Yoshida et al., 1991), which causes alcohol-related adverse physical reactions such as palpitation, nausea, headache, and general discomfort (Matsuo et al., 2006). These adverse reactions in subjects with *ALDH2*2*, which are due to excessive acetaldehyde accumulation, tend to increase alcohol sensitivity and inhibit drinking, subsequently playing a protective role against ARD. Subsequently, degrees of alcohol sensitivity regulated by the two enzymes' gene loci can be classified into the following five groups in order from the lowest alcohol sensitivity: Group I (*ALDH2 *1/*1* and *ADH1B *1/*1*), Group II (*ALDH2 *1/*1* and *ADH1B *1/*2, *2/*2*), Group III (*ALDH2 *1/*2* and *ADH1B *1/*1*), Group IV (*ALDH2 *1/*2* and *ADH1B *1/*2, *2/*2*), and Group V (*ALDH2 *2/*2* and *ADH1B *1/*1, *1/*2, *2/*2*) (Yang et al., 2010; Yokoyama et al., 2010).

Although a few studies have been conducted to determine the combined genetic effect of *ALDH2* and *ADH1B* on ARD (Kim et al., 2008; Yao et al., 2011), there is very little evidence regarding the combined effect of these two loci on internalizing mental disorders (Hishimoto et al., 2010) in spite of the strong association found between them and ARD (Baker et al., 2012; Saraceno et al., 2009). Most earlier studies on comorbidity between ARD and anxiety/depression have noted the genetic vulnerability of such internalizing disorders, and consequently focused on the candidate genes such as *DRD2 Taq* A1, *CHRM2* SNPs 5′-UTR, *5-HTT* S-allele, and *MAOA* promoter VNTR (Saraceno et al., 2009). However, genetic alcohol sensitivity can be regarded as an important reason for developing ARD. Thus, the purpose of the current study is to clarify the associations between *ALDH2* and *ADH1B* polymorphisms and depression (including suicidality) as well as anxiety disorders, with explicit assessments of ARD and drinking habit.

2. Methods

2.1. Sample

Our subjects were 2442 local government employees in the Kinki area of Japan who underwent annual health checkups from May to July, 2013. Their occupations included a variety of clerical work, along with jobs in monitoring, security, and communication services. The investigators encouraged all employees to enroll in the study, 1944 of whom (79.6%) agreed to participate in an interview survey regarding mental disorders, and provided blood samples to determine their two enzyme genetic polymorphisms. All participants gave written consent. This study was approved by the institutional review board for genetic research of Wakayama Medical University (acceptance number 106).

2.2. Psychiatric structured interview

The Mini-International Neuropsychiatric Interview (M.I.N.I.), Japanese version 5.0.0 (2003) (Sheehan et al., 1998; Sheehan and Lecrubier, 2003), a conveniently structured tool designed to identify mental disorders, was used for the present interview survey. The reliability and validity of the Japanese version of the M.I.N.I. were reported to be satisfactory (Otsubo et al., 2005). A total of 14 interviewers, all of whom were licensed doctors or nurses, were considered as competent to conduct the interviews, and enrolled. The first author (KY), a psychiatrist, trained all of them in essential interview skills, including didactic sessions of the general interview, and reviews of the instrument sections. Furthermore, the first author checked the interviewers and corrected them as the need arose during interview sessions so that the interviews could be conducted appropriately.

The M.I.N.I. deals with 17 Axis I mental disorders based on the standard of a 12-month prevalence of 0.5% or more (Sheehan et al., 1998), among which we checked the disorders listed in the first screening session: major depressive disorder, dysthymia, and suicidal risk (these three were regarded as depressive disorders). Panic disorder, agoraphobia, social phobia, specific phobia, obsessive-compulsive disorder, general anxiety disorder, and posttraumatic stress disorders (these seven were regarded as anxiety disorders) were classified as candidate depressive and anxiety disorders potentially associated with genetic alcohol sensitivity. In addition, ARD was assessed to understand the modified effect of ARD on the association between genetic alcohol sensitivity and depressive and anxiety disorders. All subjects were asked screening questions essential for the diagnosis of these disorders. If their mental symptoms satisfied those questions, more detailed questions were used to arrive at a final diagnosis of each disorder.

2.3. Genetic analysis

Genomic DNA was extracted from 1296 samples of blood using Nucleo Spin Blood Kit (Takara Bio, Otsu). Genetic determinations were made by examiners blinded to mental disorder status. TaqMan® SNP genotyping assays purchased from Applied Biosystems (Foster City, CA, USA) were used for the following (gene, SNP, assay ID): *ADH1B*, rs1229984, C_2688467_20; *ALDH2*, rs671, C_11703892_10. The remaining 648 samples were directly genotyped by the TaqMan assay on an ABI 7300 Real Time PCR System (Hayashida et al., 2014).

2.4. Statistical analysis

The *p*-value for Hardy–Weinberg equilibrium (HWE) was calculated as the difference between the number of genotypes and the number of alleles ($df = 1$). As mentioned above, the interview procedure of M.I.N.I. consists of two steps, i.e., a screening step for any mental disorders, and a detailed interview for each disorder found in the screening step. Two models were created for examining the associations between mental disorders and the two enzyme genetic polymorphisms based on the definition of outcome variables (Fig. 1). These outcomes were: (i) any depressive or anxiety disorders (final diagnoses), and (ii) any preliminary and final diagnoses of depressive or anxiety disorders. Those who did not correspond to any screening questions listed in the M.I.N.I. except ARD were categorized as normal controls ($N = 1437$). The screening question for ARD, 'In the past 12 months, have you had three or more alcoholic drinks within a three hour period on three or more occasions?,' was used as a proxy of drinking habit. 'Three or

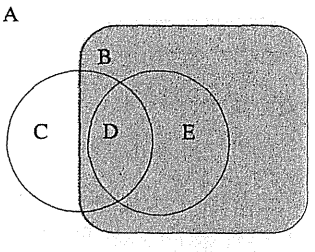

Fig. 1. Definition of outcome variables. (A) Controls without any preliminary and final diagnoses of mental disorders (n = 1437). (B) Any preliminary and final diagnoses of depressive or anxiety disorders (n = 149, Model 2). (C + D) Alcohol-related disorders (n = 168). (D + E) Any final diagnoses of depressive or anxiety disorders (n = 41, Model 1). (D) Five subjects with ARD had comorbid depressive or anxiety disorders. Those without alcohol-related disorders or any preliminary diagnoses of depressive or anxiety disorders, but with other mental disorders (including preliminary) were, excluded from the analysis (n = 211).

more alcoholic drinks' in the Japanese version means three or more glasses (three or more units on average) of any alcoholic beverage. A detailed interview was conducted on those who answered 'yes' to this screening question (i.e., drinkers) to confirm ARD as defined by DSM-IV and ICD-10. The detailed interview consisted of seven questions for alcohol dependence and four questions for alcohol abuse. Alcohol abuse was confirmed when the subjects did not meet the criteria for alcohol dependence. Those who had preliminary and final diagnoses of other mental disorders than anxiety/depressive disorders as well as ARD and did not have anxiety/depressive disorders (including preliminary) and ARD (N = 211) were excluded from the analyses.

Logistic regression analysis was used to obtain odds ratios (ORs) and 95% confidence intervals (CIs). The dependent variables in that analysis were the two outcomes defined above and ARD, which were compared with the 1437 controls. Explanatory variables were *ALDH2* and *ADH1B* genotypes classified as mentioned in Section 1 (Group I–V), adjusting for age, sex, and job rank. Age was divided into two categories, <40 or 40+, since two peaks of age distribution were observed at the dividing line of 40 years of age. Job rank was also divided into two categories, administrative position or non-administrative.

The ORs and their 95% CIs were obtained from the corresponding logistic regression coefficients and their standard errors. Each OR showed how many times subjects with the genotypes were likely to have been affected by mental disorders compared to Group IV. Group IV was set as the reference group since its members were considered better able to control their alcohol-related behaviors compared to the other lower sensitivity groups, and commonly seen in Japanese (Takeshita, 2012; Yokoyama et al., 2010). These multivariate analyses were conducted separately for drinkers and non-drinkers, as well as the entire sample. We also estimated trends of the OR in each model in accordance with the number of wild homozygotes found in the two loci. Since alcohol sensitivity has been demonstrated to be more strongly affected by *ALDH2* than *ADH1B* (Peng and Yin, 2009; Takeuchi et al., 2011), indexes related to *ALDH2*, alcohol use, and their interactive indexes adjusted for sex, age, and job rank were used to evaluate single and interactive effects of *ALDH2* on depressive and anxiety disorders in a multivariate logistic regression model. Furthermore, effects of genetic alcohol sensitivity in combination with job rank were assessed, since job rank represents the extent of job control demonstrated to be associated with depression (DeSanto Iennaco et al., 2010; Woo and Postolache, 2008). In this analysis, Group I and Group II were regarded as low alcohol sensitivity groups.

p-Values (two-sided) less than 0.05 were considered statistically significant. All computations were performed using the SAS software package, version 9.1.3 (SAS Institute, Inc., Cary, NC, USA).

3. Results

Distributions of mental disorders as well as demographic backgrounds of the subjects are shown in Table 1. Nearly 90% of the subjects were male and a little more than 10% were employed at administrative positions. The prevalence of mental disorders was low since the subjects were generally from a healthy working population. There were 41 subjects who suffered from some kind of depressive or anxiety disorder, whereas only five of them had ARD. Assuming that the proportion of those with Group I was 5.0% and the expected OR of anxiety/depressive disorders associated with this group was 5.0 based on our previous survey (Takeshita, 2012; Yoshimasu et al., 2015), the current statistical power was calculated to be 75.8% by Power and Sample Size Calculation version 3.1.2 (http://biostat.mc.vanderbilt.edu/wiki/Main/PowerSampleSize).

Table 1
Demographic backgrounds and frequencies of depressive/anxiety disorders in study subjects (N = 1944).

Variables	n (%)
Male	1742 (89.6)
Administrative position	249 (12.8)
Age (mean (SD))	37.9 (11.6)
Mental disorders	
Major depressive disorder	20 (1.0)
Dysthymia	4 (0.2)
Suicidality	13 (0.7)
Panic disorder	1 (0.05)
Agoraphobia	4 (0.2)
Specific phobia	2 (0.1)
Obsessive-compulsive disorder	8 (0.4)
Post-traumatic stress disorder	0 (0.0)
General anxiety disorders	12 (0.6)
Alcohol-related disorders[a†]	168 (8.6)
Any depressive or anxiety disorders[†]	41 (2.1)
Any depressive or anxiety disorders *without* comorbid alcohol-related disorders	36 (1.9)
Any depressive or anxiety disorders including preliminary and final diagnosis[†]	149 (7.7)

[a] Alcohol dependence and abuse.
[†] Used as outcome variables in the multiple logistic regression analyses.

Table 2
Frequencies of *ALDH2* and *ADH1B* polymorphisms and genotype classification according to the polymorphisms in study subjects (N = 1944).

ALDH2 Glu487Lys (rs671, *1 = G, *2 = A)		n (%)	
*1/*1		986 (50.7)	
*1/*2		775 (39.9)	
*2/*2		182 (9.4)	
	HWE p-value		0.10
ADH1B Arg47His (rs1229984, *1 = G, *2 = A)			
*1/*1		97 (4.9)	
*1/*2		704 (36.2)	
*2/*2		1143 (58.8)	
	HWE p-value		0.39
Genotype classification			
Group I (*ALDH2* *1/*1 and *ADH1B* *1/*1)		45 (2.3)	45 (2.3)
Group II (*ALDH2* *1/*1 and *ADH1B* *1/*2, *2/*2)		941 (48.4)	941 (48.4)
Group III (*ALDH2* *1/*2 and *ADH1B* *1/*1)		43 (2.2)	43 (2.2)
Group IV (*ALDH2* *1/*2 and *ADH1B* *1/*2, *2/*2)		733 (37.7)	733 (37.7)
Group V (*ALDH2* *2/*2 and *ADH1B* *1/*1, *1/*2, *2/*2)		182 (9.4)	182 (9.4)

HWE = Hardy–Weinberg equilibrium. Alcohol sensitivity: Group I and II: not sensitive, III: sensitive, IV: very sensitive, V: extremely sensitive.

Table 3
Numbers of each mental disorder according to *ALDH2* and *ADH1B* polymorphisms genotype classification.

Mental disorders	Group I	Group II	Group III	Group IV	Group V
Major depressive disorder	2	9	0	6	3
Dysthymia	0	1	0	2	1
Suicidality	1	4	0	6	2
Panic disorder	0	0	0	1	0
Agoraphobia	0	1	0	3	0
Specific phobia	0	0	0	1	1
Obsessive-compulsive disorder	1	2	0	4	1
Post-traumatic stress disorder	0	0	0	0	0
General anxiety disorders	0	6	0	5	1
Alcohol-related disorders[a†]	6	114	5	42	1
Any depressive or anxiety disorders[†]	4	15	0	18	4
Any depressive or anxiety disorders including preliminary and final diagnosis[†]	4	69	2	63	11

Group I (*ALDH2* *1/*1 and *ADH1B* *1/*1) $n=45$; Group II (*ALDH2* *1/*1 and *ADH1B* *1/*2, *2/*2) $n=941$; Group III (*ALDH2* *1/*2 and *ADH1B* *1/*1) $n=43$; Group IV (*ALDH2* *1/*2 and *ADH1B* *1/*2, *2/*2) $n=733$; Group V (ALDH2 *2/*2 and *ADH1B* *1/*1, *1/*2, *2/*2) $n=182$. Alcohol sensitivity: Group I and II: not sensitive, III: sensitive, IV: very sensitive, V: extremely sensitive.
† Used as outcome variables in multiple logistic regression analyses.
[a] Alcohol dependence and abuse.

Table 4
Associations between *ALDH2* and *ADH1B* polymorphisms and depressive/anxiety disorders in entire sample.

	Model 1[a] ($N=1478$)				Model 2[b] ($N=1586$)			
	Cases	Controls	OR	95% CI	Cases	Controls	OR	95% CI
Groups								
Group I	4	30	**4.32**[†]	**1.37–13.64**	4	30	1.21	0.41–3.58
Group II	15	669	0.70	0.35–1.40	69	669	0.90	0.63–1.30
Group III	0	30	NA		2	30	0.63	0.15–2.69
Group IV	18	561	1.00	Ref	63	561	1.00	Ref
Group V	4	147	0.86	0.29–2.58	11	147	0.68	0.35–1.34

Adjusted for age, sex and job rank in each model. Controls are 1437 subjects without any preliminary or final psychiatric diagnoses in all four models. NA = not available due to lack of subjects within the categories. Group I (*ALDH2* *1/*1 and *ADH1B* *1/*1); Group II (*ALDH2* *1/*1 and *ADH1B* *1/*2, *2/*2); Group III (*ALDH2* *1/*2 and *ADH1B* *1/*1); Group IV (*ALDH2* *1/*2 and *ADH1B* *1/*2, *2/*2); Group V (*ALDH2* *2/*2 and *ADH1B* *1/*1, *1/*2, *2/*2). Alcohol sensitivity: Group I and II: not sensitive, III: sensitive, IV: very sensitive, V: extremely sensitive.
[a] Any depressive or anxiety disorders, $n=1478$.
[b] Any depressive or anxiety disorders including preliminary and final diagnosis, $n=1586$.
† $p=0.013$.

The distributions of *ALDH2* and *ADH1B* polymorphisms as well as their combined classification (Group I-Group V) among all samples are shown in Table 2. No significant deviation was detected by HWE among subjects, neither for *ALDH2* nor *ADH1B*. Group II was, as expected, the most frequent, followed by Group IV. Group III had the fewest members among the five groups. These genotype distributions were consistent with the previous Japanese study (Takeshita, 2012).

Numbers of each anxiety/depressive disorder, according to *ALDH2* and *ADH1B* polymorphism combined classification (Group I–V), are shown in Table 3. All Group I subjects with any depressive or anxiety disorders at the preliminary diagnoses also had final diagnoses of such disorders ($N=4$).

Table 4 shows the association between *ALDH2* and *ADH1B* polymorphisms according to their classification, and the final and preliminary diagnoses of any anxiety/depressive disorders. Group I was significantly associated with a more than four-fold increased risk of a final diagnoses of any depressive or anxiety disorder. Group I and Group II were significantly associated with an increased risk of ARD; OR 2.97 95% CI 1.16–7.61, and OR 2.29 95% CI 1.57–3.32, respectively.

The association between Group I and V polymorphism classification and anxiety/depressive disorders was evaluated separately for drinkers and non-drinkers (Table 5). Although Group I showed non-significant, modest associations with an increased risk of such disorders among drinkers, it had a very strong (more than nine-fold increased risk) and significant association with the increased risk of a final diagnosis of anxiety/depressive disorders in non-drinkers. Trends in associations between mental disorders and the two enzyme polymorphisms, according to the number of the high-risk genotypes in the two loci (0, 1, and 2), are shown in Table 6. Trends for both models were far from statistically significant, while a significant trend for ARD was observed ($p<0.0001$).

Single and interactive effects of *ALDH2* with alcohol use on depressive and anxiety disorders are shown in Table 7. Neither *ALDH2* *1/*1 nor *ALDH2* *1/*2, nor their interactive indexes with alcohol use triggered any adverse effects increasing the risk of anxiety/depressive disorders. ARD could not be included in this model because only five subjects with anxiety/depressive disorders had comorbid ARD, too few to converge the regression model. Likewise, there were no material associations between *ADH1B* *1/*1 or *ADH1B* *1/*2 and anxiety/depressive disorders after an adjustment for sex, age, and job rank (OR 1.01 95% CI 0.45–2.27 for *ADH1B* *1/*1, OR 0.79 95% CI 0.55–1.15 for *ADH1B* *1/*2).

Table 8 shows the effects of alcohol sensitivity on anxiety/depressive disorders in combination with job rank, which is considered to reflect job control. Compared with those with high alcohol sensitivity and high job rank, those with low alcohol sensitivity and high job rank had a modest but statistically non-significant increased risk of a final diagnosis of anxiety/depression (OR = 1.85). The other combinations showed no material associations with an increased risk of such disorders.

4. Discussion

Individual alcohol sensitivity is strongly regulated by the combination of *ALDH2* and *ADH1B* genotypes. The current study for the first time applied the classification of the combined effects of the

Table 5
Associations between *ALDH2* and *ADH1B* polymorphisms and depressive/anxiety disorders among drinkers and non-drinkers.

	(*N*=1478)		Model 1[a]		(*N*=1586)		Model 2[b]	
	Cases	Controls	OR	95% CI	Cases	Controls	OR	95% CI
Drinkers[c] (*n*=1149)								
Groups								
Group I	2	22	2.73	0.55–13.59	2	22	0.82	0.18–3.72
Group II	10	463	0.59	0.24–1.43	45	463	0.79	0.48–1.28
Group III	0	19	NA		2	19	0.92	0.20–4.20
Group IV	10	277	1.00	Ref	32	277	1.00	Ref
Group V	0	15	NA		0	15	NA	
Non-drinkers[c] (*n*=795)								
Groups								
Group I	2	8	**9.20**[†]	**1.66–50.89**	2	8	2.31	0.47–11.41
Group II	5	206	0.87	0.28–2.70	24	206	1.05	0.60–1.85
Group III	0	11	NA		0	11	NA	
Group IV	8	284	1.00	Ref	31	284	1.00	Ref
Group V	4	132	1.05	0.31–3.57	11	132	0.79	0.38–1.62

Adjusted for age, sex and job rank in each model. NA = not available due to lack of subjects within the categories. Group I (*ALDH2* *1/*1 and *ADH1B* *1/*1); Group II (*ALDH2* *1/*1 and *ADH1B* *1/*2, *2/*2); Group III (*ALDH2* *1/*2 and *ADH1B* *1/*1); Group IV (*ALDH2* *1/*2 and *ADH1B* *1/*2, *2/*2); Group V (*ALDH2* *2/*2 and *ADH1B* *1/*1, *1/*2, *2/*2). Alcohol sensitivity: Group I and II: not sensitive, III: sensitive, IV: very sensitive, V: extremely sensitive.

[a] Any depressive or anxiety disorders, *n*=818 for drinkers and 660 for non-drinkers.

[b] Any depressive or anxiety disorders including preliminary and final diagnosis, *n*=877 for drinkers and 709 for non-drinkers.

[c] Those who had three glasses of alcoholic drinks within a three-hour period on three or more occasions in the past year (drinkers) or did not (non-drinkers).

[†] $p=0.011$.

Table 6
Associations between number of *1/*1 in *ALDH2* and *ADH1B*, and depressive/anxiety disorders in subjects except Group V.

	Model 1[a]		Model 2[b]	
	OR	95% CI	OR	95% CI
Groups (n of *1/*1)				
Group I (2)	**4.39**[†]	**1.39–13.89**	1.22	0.41–3.61
Group II + III (1)	0.67	0.33–1.34	0.89	0.62–1.28
Group IV (0)	1.00	Ref	1.00	Ref
p for trend	0.70		0.71	

Adjusted for age, sex and job rank in each model. Controls are 1290 subjects without any preliminary or final psychiatric diagnoses in all four models. Group I (*ALDH2* *1/*1 and *ADH1B* *1/*1); Group II (*ALDH2* *1/*1 and ADH1B *1/*2, *2/*2); Group III (*ALDH2* *1/*2 and *ADH1B* *1/*1); Group IV (*ALDH2* *1/*2 and ADH1B *1/*2, *2/*2); Group V (*ALDH2* *2/*2 and *ADH1B* *1/*1, *1/*2, *2/*2). Alcohol sensitivity: Group I and II: not sensitive, III: sensitive, IV: very sensitive, V: extremely sensitive.

[a] Any depressive or anxiety disorders, *n*=1327.

[b] Any depressive or anxiety disorders including preliminary and final diagnosis, n=1428.

[†] $p=0.012$.

Table 7
Overall associations between depressive/anxiety disorders and alcohol-related indexes.

Index	Model 1[a]		Model 2[b]	
	OR	95% CI	OR	95% CI
ALDH2 *1/*1[c]	1.17	0.34–4.12	1.43	0.68–3.01
ALDH2 *1/*2[c]	0.99	0.29–3.33	1.30	0.63–2.67
ALDH2 *1/*1 × alcohol use[d]	0.78	0.30–2.04	0.90	0.54–1.52
ALDH2 *1/*2 × alcohol use[d]	1.27	0.49–3.31	1.22	0.72–2.06
Male	0.83	0.31–2.21	0.47	0.29–0.74
Age (40+)	1.21	0.62–2.37	1.08	0.74–1.56
Job rank[e]	0.90	0.35–2.35	0.80	0.47–1.35

All variables listed above were simultaneously adjusted.

[a] Any depressive or anxiety disorders, *n*=1478.

[b] Any depressive or anxiety disorders including preliminary and final diagnosis, *n*=1586.

[c] Those with *ALDH2* *2/*2 are used as referent group.

[d] Alcohol use: those who had three glasses of alcoholic drinks within a three-hour period on three or more occasions in the past year. Nondrinkers or those with *ALDH2* *2/*2 were used as referent group.

[e] Non-administrative position.

two alcohol metabolic enzymes' polymorphisms (i.e., Group I–V) to the prevalence of depression and anxiety disorders. Compared to Group IV (*ALDH2* *1/*2 and *ADH1B* *1/*2, *2/*2), who are considered to have self-inhibition against alcohol-related behaviors, Group I (*ALDH2* *1/*1 and *ADH1B* *1/*1) and Group II (*ALDH2* *1/*1 and *ADH1B* *1/*2, *2/*2) were at a significantly elevated risk for ARD. The genotype combination of Group I also had a significant association with an increased risk of any depressive and anxiety disorders, while there were no material associations between Group II and those disorders. Such associations of Group I and depressive and anxiety disorders were more apparent in non-drinkers. Although many of the high-risk genotypes in the two loci (alleles related to low alcohol sensitivity) were significantly associated with ARD, such a trend was not observed for any depressive and anxiety disorders, including preliminary diagnoses.

On the other hand, no significant single effects were observed for both *ALDH2* and *ADH1B*, or in the interaction between *ALDH2* and alcohol use on anxiety/depressive disorders, indicating that neither *ALDH2* nor *ADH1B* by themselves have adverse effects on such mental disorders.

These findings suggest that those with low alcohol sensitivity are more likely to be affected with ARD than those with high alcohol sensitivity, consistent with previous findings (Kim et al., 2008; Yao et al., 2011; Yokoyama et al., 2010), and partially support our first hypothesis that those with low alcohol sensitivity are more likely to suffer from ARD. On the other hand, while those with *ALDH2* *1/*1 and *ADH1B* *1/*1, a very low sensitivity, were at an elevated risk for any depressive and anxiety disorders, Group II was not. Unexpectedly, this tendency was more apparent among those without actual drinking habits. Non-drinkers in Group I were confronted by a more than nine-fold increased risk of any depressive and

Table 8
Associations between depressive/anxiety disorders and genetic alcohol sensitivity in combination with job rank.

Status	Model 1[a]		Model 2[b]	
	OR	95% CI	OR	95% CI
High alcohol sensitivity[c] and high job rank[d]	1.00		1.00	
Low alcohol sensitivity and high job rank	1.85	0.33–10.35	0.93	0.38–2.24
High alcohol sensitivity and low job rank	1.32	0.30–5.74	0.78	0.40–1.53
Low alcohol sensitivity and low job rank	1.06	0.24–4.74	0.80	0.41–1.58

[a] Any depressive or anxiety disorders, $n = 1478$.
[b] Any depressive or anxiety disorders including preliminary and final diagnosis, $n = 1586$.
[c] High alcohol sensitivity: Group III (*ALDH2* *1/*2 and *ADH1B* *1/*1), Group IV (*ALDH2* *1/*2 and *ADH1B* *1/*2, *2/*2), and Group V (*ALDH2* *2/*2 and *ADH1B* *1/*1, *1/*2, *2/*2) combined. Low alcohol sensitivity: Group I (*ALDH2* *1/*1 and *ADH1B* *1/*1) and Group II (*ALDH2* *1/*1 and *ADH1B* *1/*2, *2/*2) combined.
[d] High job rank: administrative position, low job rank: non-administrative position.

anxiety disorders, compared to those of Group IV. Current findings also suggest that such adverse effects of *ALDH2* and *ADH1B* on mental health are not apparent for either of these genetic polymorphisms when acting alone.

Those with low alcohol sensitivity tended to be affected with ARD, but given that only five subjects with anxiety/depressive disorders had comorbid ARD, their depressive or anxiety symptoms, especially for those with very low sensitivity (Group I), seem not to have come directly from ARD or its related behavioral and psychosocial adversities. However, current findings are consistent with those of Hishimoto et al. (2010) in that the combination of *ALDH2* *1/*1 and *ADH1B* *1/*1 was associated with an increased risk of completed suicides, since 13 (32%) of our participants had 'suicidality'. Interestingly, in this regard, those with low alcohol sensitivity and high job rank showed a relatively higher risk for anxiety/depressive disorders (OR = 1.85). Even if they did not meet the criteria of ARD, those with high job responsibility accompanied by organizational stressors (Stuart, 2008) and low alcohol sensitivity might be more likely to indulge in drinking or to crave drinking, leading to depression or suicidal risk.

Although the reason why Group I non-drinkers suffered from depression or anxiety is unclear, there are three speculations that might explain this phenomenon. One is that those with *ALDH2* *1/*1 and *ADH1B* *1/*1 are able to relieve their mental stress adequately by moderate drinking (Marchard et al., 2003; Peele and Brodsky, 2000). However, if they cannot drink for any reason, their accumulated stress is expected to be serious enough to lead them to depression or anxiety disorders.

Another explanation is that comorbid anxiety and depressive symptoms among ARD patients might continue even after abstinence from drink. It has been reported that depression and social phobia secondary to ARD are independent conditions that do not completely remit after temperance (Olgiati et al., 2007), and abstainers have a range of characteristics known to be associated with anxiety, depression and other facets of ill health (Rodgers et al., 2000). Thus, depressive and anxiety symptoms among those who had been heavy drinkers with ARD might become conspicuous after they quit drinking.

The third possible explanation is that there is some association between genetic alcohol sensitivity and some specific behavioral patterns such as type A behavior pattern (TABP), which has been exhibited as one of the risk factors of coronary heart disease (Kuper et al., 2002; Matthews, 2005; Trigo et al., 2005). TABP as a coronary-prone behavior has been shown to be accompanied by depression or behavioral disorders (Fassino et al., 2007; Kent and Shapiro, 2009), and is associated with heavy drinking habit (Johnson et al., 1989). Since TABP is characterized by hostile, aggressive, and competitive behavior, those with TABP, *ALDH2* *1/*1 and *ADH1B* *1/*1 are expected to confront heavy mental stress when they must quit drinking. In general, and not limited to TABP, impulsivity-related personality traits such as lack of premeditation, lack of perseverance, sensation seeking, negative urgency, positive urgency, and reward sensitivity have been associated with alcohol consumption and problematic alcohol use (Stautz and Cooper, 2013). Thus, some specific personality traits linked with Group I may be proneness to depression or anxiety disorders.

We have already reported the association between the combination of *ALDH2* and *ADH1B* genotypes and entire psychiatric disorders listed in MINI (Yoshimasu et al., 2015). We conducted the current study expecting that the association between alcohol metabolizing gene and mental disorder risks would become more apparent when disorders are restricted to anxiety/depressive disorders since drinking behavior and ARD are most strongly associated with such internalizing disorders. Consequently, we excluded manic episode, eating disorders, and substance-related disorders except ARD, and reanalyzed the data. As we expected, the odds ratio of low alcohol sensitivity among non-drinkers has become higher for anxiety/depressive disorders (OR 9.20, 95% CI 1.66–50.89) compared to that for entire psychiatric disorders (OR 5.43, 95% CI 1.05–28.23). Possible speculations why such mental disorders risk increased in non-drinkers are mentioned above.

The results in this study should be interpreted according to their strengths and limitations. Two important methodological strengths are noteworthy. First, this is the first study to investigate the relationships between depression and anxiety disorders with *ALDH2* and *ADH1B* polymorphisms, unlike the majority of previous studies on this issue, which focused on ARD only. Second, we studied a large sample of employees who allowed us to conduct statistical analyses in accordance with the combination of two enzymes' genotypes (i.e., Group I–V classifications).

On the other hand, two potential limitations are noteworthy. In spite of the large sample size, there were few subjects having any depressive or anxiety disorders to be evaluated by the structured interview since our subjects were generally healthy employees, and not those recruited from patients having psychiatric disorders. Therefore, a clinically detailed evaluation of anxiety and depression was not conducted. However, the statistical power of the current sample size was calculated to be approximately 80%.

Next, the reason why non-drinking subjects with *ALDH2* *1/*1 and *ADH1B* *1/*1 did not drink was unclear. Several possible interpretations for why they suffered from depressive and anxiety disorders depended on whether they wanted to drink. For those so inclined, some depressive or anxiety disorders arose from the psychological frustration of desiring drinking, as mentioned above. However, if they disliked drinking, the mechanism explaining the associations between Group I and mental adversities would be difficult to understand, and would remain inconclusive. One speculation is that some personality traits leading to depression or anxiety might be genetically linked with the genotype combination of Group I.

In conclusion, the current study demonstrated that alcohol sensitivity regulated by combinations of *ALDH2* and *ADH1B* polymorphisms may be a useful indicator of depressive and anxiety disorders. Further larger scale, cross-cultural, clinical or

population-based studies undoubtedly will lead to a more thorough understanding of the role of gene polymorphisms related to alcohol metabolism in the development of depression and anxiety.

Author disclosures

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Contributors

Kouichi Yoshimasu is principle investigator and wrote the draft of the manuscript. Kanae Mure, Marowa Hashimoto, Mariko Hayashida, and Kenji Kinoshita conducted genetic analysis. Shigeki Takemura and Kanami Tsuno assisted the field survey. Tatsuya Takeshita gave the principle investigator special advices in interpreting the findings. Kazuhisa Miyashita superintended the entire study. All authors have read and approved the final version of the paper.

Conflict of interest

No conflict declared.

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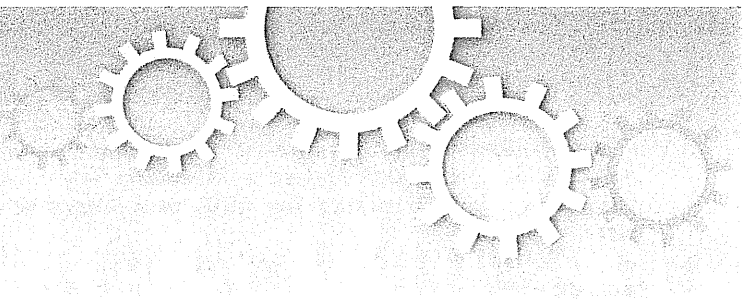

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Myeloid zinc finger 1 mediates sulindac sulfide-induced upregulation of death receptor 5 of human colon cancer cells

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A combined therapy of sulindac sulfide and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is a promising strategy for the treatment of cancer. Sulindac sulfide had been shown to induce the expression of death receptor 5 (DR5), a receptor for TRAIL, and sensitize cancer cells to TRAIL-induced apoptosis; however, the molecular mechanism underlying the upregulation of DR5 has not yet been elucidated. We demonstrate here that myeloid zinc finger 1 (MZF1) mediates the induction of DR5 by sulindac sulfide. Sulindac sulfide induced the expression of DR5 at the protein and mRNA levels in colon cancer SW480 cells. Furthermore, sulindac sulfide increased DR5 promoter activity. We showed that sulindac sulfide stimulated DR5 promoter activity via the −301 to −253 region. This region contained a putative MZF1-binding site. Site-directed mutations in the site abrogated the enhancement in DR5 promoter activity by sulindac sulfide. MZF1 directly bound to the putative MZF1-binding site of the DR5 promoter and the binding was increased by sulindac sulfide. The expression of MZF1 was also increased by sulindac sulfide, and MZF1 siRNA attenuated the upregulation of DR5 by sulindac sulfide. These results indicate that sulindac sulfide induces the expression of DR5 by up-regulating MZF1.

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is a cytokine that belongs to the TNF family[1,2] and plays an important role in immunesurveillance for cancer[3,4]. Consistent with the function of TRAIL, a TRAIL deficiency in mice was shown to accelerate carcinogenesis[5]. Previous studies have demonstrated that TRAIL selectively induced apoptosis in cancer cells both *in vitro* and *in vivo*, with little or no toxicity in normal cells[6–8]. These unique properties of TRAIL have promoted clinical trials on recombinant TRAIL and agonistic antibodies for TRAIL receptors for various malignant tumors including colorectal, renal, and ovarian cancers and melanoma[9,10]. Therefore, TRAIL is one of the most promising new candidates for cancer therapeutics. However, some tumor types have been shown to exhibit resistance to TRAIL[11]. Thus, overcoming this resistance is of importance.

TRAIL induces apoptosis through its specific receptors, death receptor 5 (DR5, also called TRAIL-R2, Apo2, TRICK2, or KILLER) and death receptor 4 (DR4), which are expressed on the cell surface[12–16]. DR5 and DR4 mediate TRAIL-induced apoptosis through interactions with adapter proteins, such as FADD, and the activation of caspases[17]. DR5 and DR4 expression levels are a limiting factor for the TRAIL sensitivity of malignant tumor cells and we and other groups have reported various types of agents that can induce the expression of DR5 and sensitize TRAIL-induced apoptosis in malignant tumor cells[18,19]. To overcome the resistance of malignant tumors to TRAIL therapy, we previously proposed a strategy combining TRAIL and a DR5 inducer[19], because the amount of DR5 is known to regulate TRAIL sensitivity in cancer cells.

Sulindac sulfide is one of the nonsteroidal anti-inflammatory drugs (NSAIDs). Although NSAIDs have generally been used to treat pain, inflammation, and fever, the continuous usage of NSAIDs has been shown to reduce the risk of various cancers, especially colon cancer[20–22], and metastasis in patients who subsequently develop cancer[23].

A single treatment with sulindac sulfide inhibited the growth of cancer cells by inducing apoptosis and necrosis through gadd45a upregulation[24] and phosphodiesterase 5 inhibition[25]. Sulindac sulfide has also been shown to sensitize cancer cells to TRAIL-induced apoptosis via the upregulation of DR5[26,27], which indicates that sulindac sulfide is a promising candidate for overcoming TRAIL resistance in cancer cells. However, the precise mechanism by which sulindac sulfide up-regulates DR5 remains unclear.

In the present study, we demonstrated for the first time that sulindac sulfide up-regulated DR5 through the transcription factor myeloid zinc finger 1 (MZF1) and overcame TRAIL-induced apoptosis in cancer cells.

Results

Sulindac sulfide enhanced TRAIL-induced apoptosis in SW480 colon cancer cells. We examined the effect of sulindac sulfide on TRAIL-induced apoptosis by measuring the sub-G1 population, which reflected hypodiploid cells. Sulindac sulfide or TRAIL alone slightly induced apoptosis in SW480 colon cancer cells; however, the combined treatment with sulindac sulfide and TRAIL markedly induced apoptosis (Fig. 1a). As a result of DAPI staining, the combined treatment with sulindac sulfide and TRAIL induced condensed nuclei (Fig. 1b). The combined effect was blocked by the pan-caspase inhibitor zVAD-fmk, which indicated that apoptosis was caspase-dependent (Fig. 1c). Moreover, the combination cleaved caspase-8, caspase-3 and PARP (Fig. 1d). These results indicate that sulindac sulfide enhanced the efficacy of TRAIL to induce apoptosis and overcame TRAIL resistance in SW480 colon cancer cells.

Sulindac sulfide induced DR5 expression in SW480 cells. We next examined whether sulindac sulfide affected gene expression related to cell death using the RNase protection assay. As shown in Figure 2a, sulindac sulfide increased DR5, DR4, and TRAIL mRNA levels. Among these, the upregulation of DR5 by sulindac sulfide was the most prominent. Therefore, we confirmed that sulindac sulfide increased DR5 mRNA level in a dose-dependent manner by Northern blotting (Fig. 2b). Sulindac sulfide also increased DR5 protein expression in a dose- and time-dependent manner (Fig. 2c

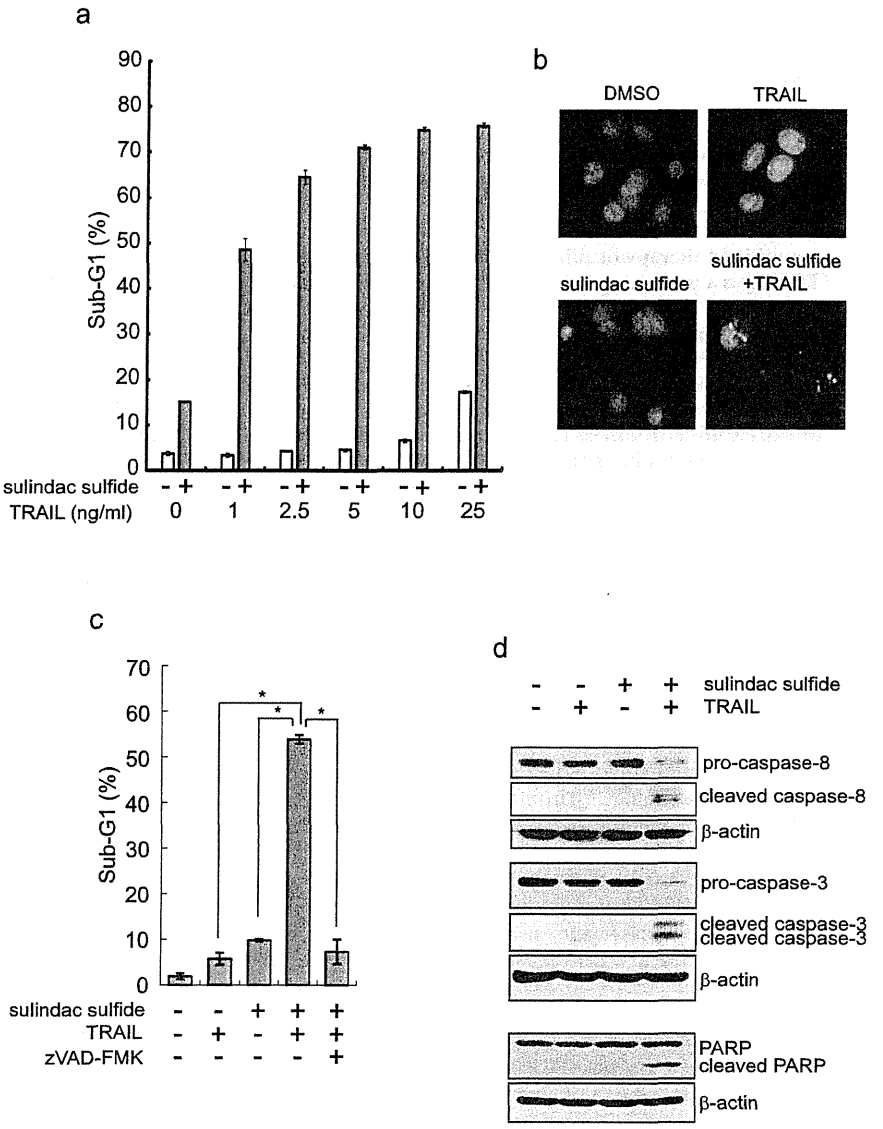

Figure 1 | Sulindac sulfide enhanced TRAIL-induced apoptosis in SW480 cells. (a) SW480 cells were treated with 200 μM sulindac sulfide and/or the indicated concentrations of TRAIL for 24 h. Cells were analyzed for DNA content by PI staining (FL2-H) using a flow cytometer. The percentages of sub-G1 are shown as a bar graph. (b) DAPI staining of SW480 cells. SW480 cells were treated with 200 μM sulindac sulfide and/or 10 ng/ml TRAIL for 24 h. Nuclear morphology was visualized using DAPI staining under a fluorescence microscope. (c) SW480 cells were treated with 200 μM sulindac sulfide and/or 10 ng/ml TRAIL with or without 20 μM zVAD-fmk for 24 h. The effects were analyzed as described in (a). Data represent the means +/− S.D. of three determinations. *: $p < 0.05$ (d) Western blotting for caspase-8, caspase-3 or PARP. SW480 cells were treated with 200 μM sulindac sulfide and/or 10 ng/ml TRAIL for 24 h. β-actin was used as a loading control.