Ethics Committee at Nagoya City University Medical School.

In the present study: (1) the prevalence and the association among 4 aPL were examined in 367 patients; (2) subsequent pregnancy outcome after systematic examination for pregnancy loss was determined in patients who received no medication or anticoagulants, comparing patients with and without aPE; (3) subsequent pregnancy outcome was examined excluding cases with abnormal karyotypes in aborted concepti; and (4) AUCs for ROC curves of aPE were calculated and receiver operating characteristic (ROC) analysis was carried out to ascertain whether aPE have predictive value for further miscarriage in 181 recurrent cases which received no medication.

2.2. Modified assays for the IgG and IgM isotypes against plasma protein binding phosphatidylethanolamine complex or phosphatidylethanolamine alone

Briefly, microtiter plates were coated with 30 μl of 50 μg/ml of PE (Aventi Polar Lipids, Birmingham, AL, USA), and each well was blocked for 1 h with 10% bovine serum albumin. To detect phospholipid-binding plasma protein dependent and independent reactivity, 50 μl aliquots of patient plasma diluted 1:100 containing either 10% adult bovine plasma or 1% bovine serum albumin were incubated for 1 h. Antibodies were detected with alkaline phosphatase labeled anti-human IgG or IgM antibodies. Nonspecific binding control wells were processed in parallel and the background values were subtracted (Sugi et al., 1999).

Cut-off levels were set at mean ± 2SD, established using sera from 122 healthy volunteers. Therefore 0.32, 0.45, 0.44 and 1.0 were considered positive for P+ aPE IgG, P − aPE IgG, P + aPE IgM and P − aPE IgM, respectively.

2.3. Assay for the lupus anticoagulant by the diluted aPTT method

Brain cephalin (Automated aPTT, Organon Teknica, Durham, NC) was employed as a phospholipid reagent for the determination of aPTT, diluted 5 times in veronal saline (Ogasawara et al., 1996a).

A: Fifty μl of non-pregnant control woman plasma, 50 μl of standard plasma, and 100 μl of diluted cephalin were mixed and incubated for exactly 3 min at 37 °C. B: At the same time 100 μl of standard plasma alone and 100 μl of diluted cephalin were mixed and incubated for 3 min at 37 °C. One hundred microlitres of 25 mM $CaCl_2$ was added and clotting time was measured with an Option 4 bioMeriux calculator, France.

Clotting times for A–B with control plasma samples from 104 healthy non-pregnant women were first examined. The mean and standard deviation values were 2.57 and 1.60 s, respectively. Lupus anticoagulant was considered positive when prolonged clotting times (>mean ± 3SD, 7.37 s) failed to correct when samples were mixed 1:1 with standard plasma.

2.4. Assay for the lupus anticoagulant with reference to the diluted Russell's viper venom time

To determine *T*1, 200 μl of healthy non-pregnant control woman plasma and 200 μl of diluted Russell viper venom and phospholipid reagents containing 25 mM $CaCl_2$ (Gradipore Ltd., Pyrmont, Australia) were mixed and clotting time was measured with an Option 4 bioMeriux calculator, France. To determine *T*2, 200 μl of the same non-pregnant control plasma and 200 μl of diluted Russell's viper venom and phospholipid-rich reagents containing 25 mM $CaCl_2$ were mixed and clotting time was measured. The mean and standard deviation values were 0.9 and 0.1 s, respectively. Lupus anticoagulant was considered positive when *T*1/*T*2 was over 1.3.

2.5. Modified assays for the IgG isotypes of β2GPI-dependent and -independent aCLs

Briefly, cardiolipin in ethanol (2.5 μg/50 μl/well) was coated onto the surfaces of polystyrene microtiter plates by evaporation under nitrogen. For the detection of β2GPI-dependent aCL, duplicate wells were incubated with 50 μl of HEPES–BSA, containing purified human β2GPI (30 μg/ml; Yamasa Corp., Choshi, Japan), for 10 min at room temperature. For the determination of β2GPI-independent aCL, duplicate wells were incubated with 50 μl of HEPES–BSA in the same manner. Fifty microlitres of test sera, diluted 1:202 in HEPES–BSA, were then introduced into the duplicate wells and incubations were performed for 30 min at room temperature. After washing with PBS–Tween, wells were exposed to 100 μl of horseradish peroxidase-labeled murine monoclonal IgG against human IgG (G-02; Yamasa Corp.) for 30 min at room temperature. After washing, a 100 μl aliquot of 0.3 mM tetramethylbendizine solution containing 0.003% of H_2O_2 was added to each well. The reaction was terminated by adding 100 μl of 2N H_2SO_4, and the optical density was measured at 450 nm. Antibody titers (units/ml) of aCL were calculated from a standard curve, obtained by running six calibration standards (Yamasa Corp., 1.3–125 units/ml) for each plate.

Test results for β2GPI-dependent and -independent aCL were considered positive when the antibody level was above the 99% confidence interval for 283 normal non-pregnant control sera. This was more than 1.9 units/ml for β2GPI-dependent and more than 6.3 units/ml for β2GPI-independent aCL. In addition, in order to avoid false positives due to nonspecific binding, a β2GPI-dependent assay had to show a higher value than the β2GPI-independent assay performed in parallel, to be considered positive (Matsuura et al., 1994; Katano et al., 1996).

2.6. Statistical analysis

Receiver operating characteristics (ROC) curves for each PE were drawn for all cut-off points. In order to examine the diagnostic values for each PE, areas under the curves (AUCs) for ROC curves were calculated. The analysis was carried out with the PROC LOGISTIC procedure in SAS sys-

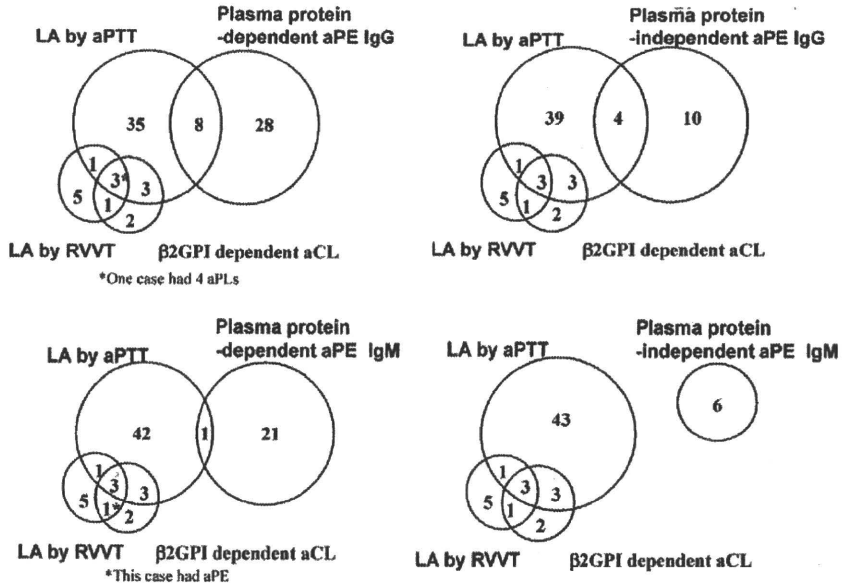

Fig. 2. Overlapping associations among the 4 aPLs for the 367 patients with a history of recurrent pregnancy loss.

tem version 9.1 (SAS Institute Inc., NC, USA) with $P < 0.05$ considered to be statistically significant.

3. Results

Totals of 37 (10.1%), 14 (3.8%), 23 (6.3%), 6 (1.6%), 9 (2.5%), 10 (2.7%) and 50 (13.6%) of the 367 patients were, respectively, positive for P+aPE IgG, P−aPE IgG, P+aPE IgM, P−aPE IgM, β2GPI-dependent aCL, lupus anticoagulant by RVVT and lupus anticoagulant by aPTT.

The relations among aPE and conventional aPL are shown in Fig. 2. Patients with P+aPE IgG were separated from those with β2GPI-dependent aCL and lupus anticoagulant by RVVT. Only one case was positive for all tests. Eight of 37 patients had both P+aPE IgG and lupus anticoagulant by aPTT. On the other hand, only one patient had P+aPE IgM and LA. Six patients with P−aPE IgM were completely separated from conventional aPL.

Eighty-eight of 367 (24.0%) patients miscarried again. Characteristics and subsequent pregnancy outcome for all 302 patients who received no medication or anticoagulant are given in Table 1. With regard to the no medication group, 10 of 14 patients (71.4%) positive for P+aPE IgG gave birth to living babies, while 127 of 167 patients (76.0%) negative for P+aPE IgG had successful pregnancies (difference not significant). A total of 4 of 7 patients (57.1%) positive for P−aPE IgG gave birth to living babies, while 133 of 174 patients (76.4%) negative for P−aPE IgG had successful pregnancies (difference not significant).

Fifty-five karyotypes of miscarried concepti could be analyzed and 31 (56.4%) were found to be abnormal. After excluding miscarriage cases caused by an abnormal embryonal karyotype, the success rate (83.3%) of patients positive did not differ from that (83.6%) of patients negative for P+aPE IgG.

AUCs for each ROC curve, as shown in Fig. 2, for P+aPE IgG, P−aPE IgG, P+aPE IgM and P−aPE IgM was 0.535, 0.612, 0.546 and 0.533, respectively. Each AUC was close to 0.5 so that there was no variation in diagnostic capacity of the test. These results thus did not suggest any significant predictive value of 4 aPE for further miscarriage (Fig. 3).

4. Discussion

In the present study, the population with aPE was found to differ substantially from those with β2GPI-dependent aCL and lupus anticoagulant by RVVT. Only 8 patients had both P+PE IgG and lupus anticoagulant by aPTT. It is well-known that purified IgG from patients with lupus anticoagulant has lupus anticoagulant activity, thus patients with lupus anticoagulant by aPTT had not aPE IgM but aPE IgG in the present study. aPTT influences the intrinsic pathway including the contact phase cascade and, in contrast, RVVT inhibits coagulation factor X directly. Lupus

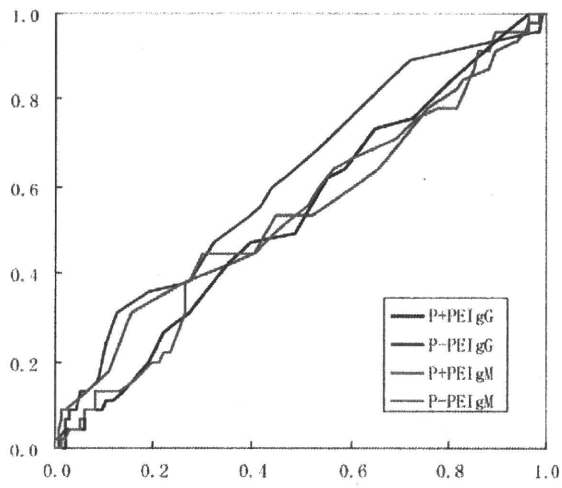

Fig. 3. ROC curves for anti-PE antibodies.

Table 1
Characteristics and pregnancy outcome of patients with antiphosphatidylethanolamine antibodies.

	No medication		Anticoagulant	
	Positive	Negative	Positive	Negative
P + aPE IgG	$n=14$	$n=167$	$n=12$	$n=109$
Mean age	31.3	31.6	34.9	32.6
Mean no. of previous losses	2.2	2.3	3.0	3.1
Failure	4 (2)	40 (25)	4 (2)	26 (17)[a]
Success	10	127	8	83
Success rate (%)	71.4 (83.3)	76.0 (83.6)	66.7 (80.0)	76.1 (83.0)[b]
P – aPE IgG	$n=7$	$n=174$	$n=5$	$n=116$
Mean age	33.0	31.5	32.0	37.8
Mean no. of previous losses	2.0	2.3	3.0	3.1
Failure	3 (1)	41 (26)	2 (2)	28 (17)
Success	4	133	3	88
Success rate (%)	57.1 (80.0)	76.4 (83.6)	60.0 (60.0)	75.9 (83.8)
P + aPE IgM	$n=6$	$n=175$	$n=11$	$n=110$
Mean age	31.7	31.5	32.2	32.5
Mean no. of previous losses	2.0	2.3	3.0	3.2
Failure	4 (3)	40 (24)	2 (1)	28 (18)
Success	2	135	9	82
Success rate (%)	33.3 (40.0)	77.1 (84.9)	81.8 (90.0)	74.5 (82.0)
P – aPE IgM	$n=2$	$n=179$	$n=2$	$n=119$
Mean age	30.5	31.4	29.5	32.7
Mean no. of previous losses	3.0	2.0	2.5	3.1
Failure	0	44 (27)	0	30 (19)
Success	2	135	2	89
Success rate (%)	100 (100)	75.4 (83.3)	100	74.8 (82.4)

[a] Miscarriages caused by an abnormal embryonal karyotype were excluded.
[b] Success rate when miscarriages caused by an abnormal embryonal karyotype were excluded.

anticoagulant comprises heterogeneous antibodies against phospholipid-binding prothrombin, factor X and/or β2GPI (Bever et al., 1991; Brandt, 1991; Roubey et al., 1992). Thus, lupus anticoagulant by aPTT included β2GPI-dependent aCL and lupus anticoagulant by RVVT. aPE can recognize kininogen–PE complexes (Sugi and McIntyre, 1995). Lupus anticoagulant acting by aPTT but not by RVVT might comprise IgG against kininogen–PE complexes.

Antigenic targets include β2GPI, prothrombin, high and low molecular weight kininogen, annexin V, protein C and protein S (Roubey et al., 1992; Roubey, 1994). In addition to approaches for conventional aPLs, new ELISA methods for aPE, anti-prothrombin and anti-annexin V antibodies are now available (Bever et al., 1991; Sugi and McIntyre, 1995; Matsubayashi et al., 2001). We have shown that β2GPI-dependent aCL is a strong predictor of intrauterine fetal death, intrauterine growth restriction and pregnancy-induced hypertension, although the frequency is low (Katano et al., 1996). These conventional aPLs are included in the international criteria for APS. However, the prevalence of β2GPI-dependent aCL and lupus anticoagulant by dRVVT are relatively low (2.5 and 2.7% in the present study) and Sugi et al. (1999) have concluded that aPEs are more strongly associated with recurrent pregnancy loss because the prevalence of PE IgG and IgM were found to be much higher (20.1 and 12.2%).

However, a high prevalence in a particular test does not necessarily imply clinical significance. With regard to antinuclear antibodies (ANA), the frequency is significantly higher than in controls, but no effects on the live birth rate were found in one study (Ogasawara et al., 1996b).

Moreover, it is unlikely that all these molecules are targeted at the same time; the whole situation rather reflects the extensive immunologic alterations that characterize the pregnant status. The exact role of even β2GPI itself in pregnancy loss remains unknown because knockout mice are fertile (Miyakis et al., 2004).

To our knowledge there have hitherto been only a few reports of the predictive value of aPE for adverse pregnancy outcome of recurrent aborters. Gris et al. (2000) described aPE IgM but not IgG to have predictive value for subsequent fetal loss from the 8th week up to and including the 22nd week of gestation, in spite of low dose aspirin treatment. Recently, Yamada et al. (2009) measured aPLs including aPE IgG during the first trimester in a consecutive series of 1155 pregnant women and found that aPE IgG was associated with developing pregnancy-induced hypertension (8.3, 2.4–29) and premature delivery (12.7, 3.1–50). However, they could not examine the association between aPE IgG and early miscarriage because the peripheral blood was obtained at 8–14 weeks' gestation.

Another issue that relates to aPE and most of the aPL is the lack of standardization and of uniform requirements for performance and interpretation of the tests. From comparing the detection methods for aPE in the present study with those in Gris's study, there are some important differences (for example, regarding overnight incubation and methanol dilutions of aPE plates, extraction of standards for OD estimation). Similar limitations and particularities exist for the measurements of most of the aPLs. Using different detection methodologies can cause different prevalence values, even though the same aPL are purportedly mea-

sured. Another limitation is that we did not measure persistent aPE though International criteria recommend two times measurement.

Our present study, the first to examine the predictive value of aPE in recurrent pregnancy loss patients, failed to prove any significant link with an adverse outcome in untreated patients. We could not examine subsequent pregnancy loss of the 8 cases with both P+aPE IgG and lupus anticoagulant by aPTT without medication and we have proved that the live birth rate in patients with a history of recurrent miscarriage who had lupus anticoagulant by aPTT was improved from 46.2 to 80.4% by anticoagulant therapy (Ogasawara et al., 2001). It is unclear whether P+aPE IgG might have a predictive value if the 8 patients with no medication were followed up. This is not a case–control study but a cohort study. Patients with APS, occasional aPL and unexplained causes were treated with anticoagulant. Thus, it is difficult to make a simple direct comparison between the two groups with and without anticoagulant. Further study is needed excluding the influence of lupus anticoagulant by aPTT because lupus anticoagulant by aPTT cannot be examined commercially, although tests for aPE, β2GPI-dependent aCL and lupus anticoagulant by dRVVT are commercially obtainable and widely applied in Japan.

The prevalence of aPE IgG and aPE IgM were earlier reported to be 20.1 and 12.2% (Sugi et al., 1999). The prevalence of aPE IgG included P+aPE IgG and P−aPE IgG and the prevalence of P+aPE IgG and P+aPE IgM were 11.5 and 5.8%, respectively. These were 10.1 and 6.3% in the present study, where we performed ELISA using not purified kininogen but rather bovine plasma obtainable commercially. We should focus on kininogen dependence because a previous study showed a link with kininogen–PE IgG (Sugi and McIntyre, 1995; Sugi et al., 1999). The contact activation system consists of coagulation factor XII, kallikrein and high molecular weight kininogen. Factor XII can be activated by contact with negatively charged surfaces and factor XIIa then converts prekallikrein to kallikrein. Kallikrein digests kininogen to liberate the vasoactive, proinflammatory mediator, bradykinin. Factor XIIa also stimulates factor XI, which activates the intrinsic coagulation pathway. Decreased factor XII activity is also associated with recurrent miscarriage (Ogasawara et al., 2001). The presence of low molecular weight kininogen and high molecular weight kininogen in the porcine uterus and endometrial gene expression of plasma kallikrein and factor XII provide evidence that the kallikrein–kininogen–kinin system is biologically active during establishment of pregnancy in the pig (Vonnahme et al., 2004). Alternatively, kininogen-dependent PE IgG might thus be associated with early spontaneous abortion by the inhibition of bradykinin-induced relaxation in uteral arteries (Kenny et al., 2002).

In conclusion, we did not obtain any evidence that aPE predicts further miscarriage in recurrent pregnancy loss patients. However, a further study is needed for confirmation because the sample size of the population with no medication was too small. Improvements are also required for the commercially obtained assay of aPE in common use in Japan and an investigation is clearly warranted to exclude influence of lupus anticoagulant by aPTT because it identifies a different population from those with conventional aPL.

Acknowledgment

The authors thank Hiroko Johnokuchi (Department of Food Science and Nutrition, Faculty of Human Life and Environmental Sciences, Nagoya Women's University) for her technical support.

References

Bever, E.M., Galli, M., Barbui, T., Comfurius, P., Zwaal, R.F.A., 1991. Lupus anticoagulant IgG's (LA) are not directed to phospholipids only, but to a complex of lipid-bound human prothrombin. Thromb. Haemost. 66, 629–632.

Brandt, J.T., 1991. Assays for phospholipid-dependent formation of thrombin and Xa: a potential method for quantifying lupus anticoagulant activity. Thromb. Haemost. 66, 453–458.

Farquharson, R.G., Pearson, J.F., John, L., 1984. Lupus anticoagulant and pregnancy management. Lancet 28, 228–229.

Gris, J.C., Quéré, I., Sanmarco, M., Boutiere, B., Mercier, E., Amiral, J., Hubert, A.M., Ripart-Neveu, S., Hoffet, M., Tailland, M.L., Rousseau, O., Monpeyroux, F., Dauzat, M., Sampol, J., Daures, J.P., Berlan, J., Marès, P., 2000. Antiphospholipid and antiprotein syndromes in non-thrombotic, non-autoimmune women with unexplained recurrent primary early foetal loss. The Nimes Obstetricians and Haematologists Study—NOHA. Thromb. Haemost. 84, 228–236.

Katano, K., Aoki, A., Sasa, H., Ogasawara, M., Matsuura, E., Yagami, Y., 1996. Beta 2-glycoprotein I-dependent anticardiolipin antibodies as a predictor of adverse pregnancy outcomes in healthy pregnant women. Hum. Reprod. 11, 509–512.

Katano, K., Aoki, K., Ogasawara, M.S., Suzumori, K., 2000. Adverse influence of numbers of previous miscarriages on results of paternal lymphocyte immunization in patients with recurrent spontaneous abortions. Am. J. Reprod. Immunol. 44, 289–292.

Kenny, L.C., Baker, P.N., Kendall, D.A., Randall, M.D., Dunn, W.R., 2002. Differential mechanisms of endothelium-dependent vasodilator responses in human myometrial small arteries in normal pregnancy and pre-eclampsia. Clin. Sci. 103, 67–73.

Matsubayashi, H., Arai, T., Izumi, S., Sugi, T., McIntyre, J.A., Makino, T., 2001. Anti-annexin V antibodies in patients with early pregnancy loss or implantation failures. Fertil. Steril. 76, 694–699.

Matsuura, E., Igarashi, Y., Fujimoto, M., Ichikawa, I., Koike, T., 1990. Anticardiolipin cofactor(s) and differential diagnosis of autoimmune disease. Lancet 21, 177–178.

Matsuura, E., Igarashi, Y., Yasuda, T., Triplett, D.A., Koike, T., 1994. Anticardiolipin antibodies recognize β2-glycoprotein I structure altered by interacting with an oxygen modified solid phase surface. J. Exp. Med. 179, 457–462.

Miyakis, S., Robertson, S.A., Krillis, S.A., 2004. Beta-2 glycoprotein I and its role in antiphospholipid syndrome-lessons from knockout mice. Clin. Immunol. 112, 136–143.

Miyakis, S., Lockshin, M.D., Atsumi, T., Branch, D.W., Brey, R.L., Cervera, R., Derksen, R.H.W.M., De Groot, P.G., Koike, T., Meroni, P.L., Reber, G., Shoenfeld, Y., Tincani, A., Vlachoyiannopoulos, P.G., Krilis, S.A., 2006. International consensus statement of an update of the classification criteria for definite antiphospholipid syndrome (APS). J. Thromb. Haemost. 4, 295–306.

Ogasawara, M., Aoki, K., Matsuura, E., Sasa, H., Yagami, Y., 1996a. Anti β2glycoprotein I antibodies and lupus anticoagulant in patients with recurrent pregnancy loss: prevalence and clinical significance. Lupus 5, 587–592.

Ogasawara, M., Aoki, K., Kajiura, S., Yagami, Y., 1996b. Are antinuclear antibodies predictive of recurrent miscarriage? Lancet 347, 1183–1184.

Ogasawara, M., Aoki, K., Katano, K., Ozaki, Y., Suzumori, K., 2001. Factor XII but not protein C, protein S, antithrombin III or factor XIII as a predictor of recurrent miscarriage. Fertil. Steril. 75, 916–919.

Rai, R., Cohen, H., Dave, M., Regan, L., 1997. Randomised controlled trial of aspirin and aspirin plus heparin in pregnant women with recurrent miscarriage associated with phospholipid antibodies (or antiphospholipid antibodies). BMJ 314, 253–257.

Roubey, R.A.S., Pratt, C.W., Buyon, J.P., Winfield, J.B., 1992. Lupus anticoagulant activity of autoimmune antiphospholipid antibodies is dependent upon β2glycoprotein I. J. Clin. Invest. 90, 1100–1104.

Roubey, R.A.S., 1994. Autoantibodies to phospholipid-binding plasma proteins: a new view of lupus anticoagulant and other "antiphospholipid" autoantibodies. Blood 84, 2854–2867.

Sugi, T., McIntyre, J.A., 1995. Autoantibodies to phosphatidylethanolamine (PE) recognize a kininogen–PE complex. Blood 86, 3083–3089.

Sugi, T., Katsunuma, J., Izumi, S., McIntyre, J.A., Makino, T., 1999. Prevalence and heterogeneity of antiphosphatidylethanolamine antibodies in patients with recurrent early pregnancy losses. Fertil. Steril. 71, 1060–1065.

Sugiura-Ogasawara, M., Ozaki, Y., Sato, T., Suzumori, N., Suzumori, K., 2004. Poor prognosis of recurrent aborters with either maternal or paternal reciprocal translocation. Fertil. Steril. 81, 367–373.

Sugiura-Ogasawara, M., Ozaki, Y., Kitaori, T., Kumagai, K., Suzuki, S., 2010. Midline uterine defect size correlated with miscarriage of euploid embryos in recurrent cases. Fertil. Steril. 93, 1983–1988.

Yamada, H., Atsumi, T., Kobashi, G., Ota, C., Kato, E.H., Tsuruga, N., Ohta, K., Yasuda, S., Koike, T., Minakami, H., 2009. Antiphospholipid antibodies increase the risk of pregnancy induced hypertension and adverse pregnancy outcomes. J. Reprod. Immunol. 79, 188–195.

Vonnahme, K.A., Fernando, S.C., Ross, J.W., Ashworth, M.D., DeSilva, U., Malayer, J.R., Geisert, R.D., 2004. Porcine endometrial expression of kininogen, factor XII, and plasma kallikrein in cyclic and pregnant gilts. Biol. Reprod. 70, 132–138.

Regular Article

Psychotherapy and Psychosomatics

Psychother Psychosom 2010;79:303–311
DOI: 10.1159/000319311

Received: October 30, 2009
Accepted after revision: March 11, 2010
Published online: July 28, 2010

Depression, Alexithymia and Long-Term Mortality in Chronic Hemodialysis Patients

Masayo Kojima[a] Junichiro Hayano[b] Sadao Suzuki[a] Hachiro Seno[d]
Hirotake Kasuga[d] Hiroshi Takahashi[f] Takanobu Toriyama[e]
Hirohisa Kawahara[d–f] Toshiaki A. Furukawa[c]

Departments of [a]Public Health, [b]Medical Education and [c]Psychiatry and Cognitive-Behavioral Medicine, Nagoya City University Graduate School of Medical Sciences, and Departments of [d]Nephrology and [e]Cardiology and [f]Division of Outcome Research, Nagoya Kyoritsu Hospital, Nagoya, Japan

Key Words
Alexithymia · Depression · Epidemiology · Mortality · Prognostic factors · Hemodialysis

Abstract
Background: Depression increases the risk of mortality in hemodialysis patients. Alexithymia, a disorder of affect regulation, has also been reported to be associated with mortality risk in the general population. We conducted a prospective study to estimate the independent impact of depression and alexithymia on long-term mortality. ***Methods:*** A total of 230 hemodialysis outpatients, with a mean age of 56.3 ± 9.6 years, completed a batch of self-report measures including the Beck Depression Inventory-II (BDI-II), the 20-item Toronto Alexithymia Scale (TAS-20) and the 36-item Short Form Health Survey (SF-36). Survival status was confirmed every 6 months for up to 5 years. The presence of depression and alexithymia was defined by a BDI-II score of ≥14 and a TAS-20 score of ≥61, respectively. ***Results:*** During the follow-up period, 27 deaths were confirmed. Both depression and alexithymia were associated with an increased risk for all-cause mortality; the age- and sex-adjusted hazard ratio for depression was 2.36 (95% CI: 1.08–5.15; p = 0.03) and that for alexithymia was 4.29 (95% CI: 1.95–9.42; p < 0.001). Depression lost its statistical significance after controlling for alexithymia, whereas alexithymia remained significant even after adjusting for the baseline depression, health status (the summary scores of the SF-36), marital status and clinical covariates (multivariate adjusted hazard ratio = 3.62; 95% CI: 1.32–9.93; p = 0.01). ***Conclusions:*** Alexithymia is a strong independent risk factor for all-cause mortality in hemodialysis patients.

Introduction

Patients with end-stage renal disease (ESRD) are at great risk for developing emotional disturbances due to the burden from illness, such as time constraints, diet restrictions, functional limitations, changes in self-perception, and fear of death [1, 2]. Depressive symptoms have been suggested to exert an influence on the prognosis of various diseases and mortality in the clinical and general population [3]. A positive association between depression

KARGER

Fax +41 61 306 12 34
E-Mail karger@karger.ch
www.karger.com

0033–3190/10/0795–0303$26.00/0

Accessible online at:
www.karger.com/pps

Masayo Kojima
Department of Public Health
Nagoya City University Graduate School of Medical Sciences
1 Kawasumi, Mizuho-cho, Mizuho-ku, Nagoya 467-8601 Japan
Tel. +81 52 853 8176, Fax +81 52 842 3830, E-Mail masayok@med.nagoya-cu.ac.jp

and increased mortality has also been reported in ESRD patients [4–7].

Alexithymia, a personality construct that reflects a deficit in the cognitive processing of emotion [8], has been noted by psychotherapists as a common characteristic in classic psychosomatic patients for whom therapy was unsuccessful [9, 10]. Alexithymic individuals tend to have difficulty identifying and describing their inner feelings, to fantasize rarely and to have a utilitarian thinking style [11]. Researchers have revealed a strong linkage between alexithymia and various mental and physical health problems [12–15]. Some investigators have suggested that alexithymia may interfere with treatment compliance, influence treatment outcomes in clinical settings, and increase mortality risk in the general population [16, 17]. Recently, we found that alexithymia was independently associated with the presence of and deterioration in depression in hemodialysis patients [18]. However, whether alexithymia influences long-term prognosis in this population has not been examined.

The purpose of the current prospective study was to examine the association of depression and alexithymia with 5-year mortality in hemodialysis patients with ESRD. We hypothesized that both depression and alexithymia would be independently associated with increased 5-year mortality.

Subjects and Methods

Subjects

This prospective multicenter study was designed to determine the influence of psychosocial factors on the long-term prognosis of ESRD patients. The research ethics committee of the Nagoya City University Graduate School of Medical Sciences, Japan, approved the research protocol. The details of the study methods are described in detail elsewhere [19]. Briefly, the study subjects were recruited from ESRD patients who had received regular hemodialysis therapy (i.e. one 4-hour hemodialysis session 3 times per week) at any of 3 clinics in Japan (the Nagoya Central Clinic, the Anjoh Central Clinic and the Hekikai Central Clinic) between May 2001 and May 2002. We selected patients who were less than 70 years old and could read and complete the self-administered questionnaire unaided. To avoid the transitional influences of suffering from life-threatening states on psychological factors, we excluded those who had experienced episodes of acute myocardial infarction or stroke, those with a major surgical procedure within the past 2 months, or those with a malignant neoplasm or any psychiatric diagnosis within the past 5 years. Of the 538 patients registered in the 3 clinics, 207 were excluded for the following reasons: 132 patients were aged 70 years or above, 32 patients had visual or cognitive difficulties in completing the questionnaire, 29 had severe medical histories, and 14 were moved or had died before the interview. Out of 331 eligible patients, 21 moved out of the study area before completing the baseline survey, 8 could not complete the questionnaire due to illness, and 72 refused to participate in the study. A total of 230 patients provided written informed consent for participation in the study and completed the questionnaires.

Procedures

At the time of enrollment, trained research assistants conducted baseline interviews and assessed the participants' demographic characteristics and medical histories. The medical data obtained from hospital charts included the serum concentration of albumin, phosphorus, calcium, protein catabolic rate (PCR) and Kt/V, which are established indices associated with survival in hemodialysis patients [19–22]. Interdialytic weight gain was calculated as the patients' weight at the beginning of the hemodialysis session minus the weight after the previous hemodialysis session divided by the nephrologist-determined dry weight, divided by the interdialytic period in days [23]. All of the participants completed a battery of well-validated self-reporting inventories described below for psychological evaluation. These self-report measures were translated into Japanese using the back-translation method [24–27].

Self-Report Measures

Beck Depression Inventory-II. The second edition of the Beck Depression Inventory-II (BDI-II) [28] was used to assess the level of depressive symptoms. The BDI is the most popular self-reporting tool for measuring depressive symptoms and has often been used to evaluate mental health in ESRD patients [29]. It was revised to correspond to the Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition, criteria and was published as the BDI-II in 1996. The BDI-II scores range from 0 to 63, and a cutoff score of 14 indicates at least a mild-to-moderate level of depression.

20-Item Toronto Alexithymia Scale. The 20-item Toronto Alexithymia Scale (TAS-20) was used to evaluate alexithymia [30, 31]. The total TAS-20 scores range from 20 to 100, and a score of 61 or higher was suggested for use in alexithymia screening by the original authors [31].

Social Support Questionnaire. The 6-item version of the Social Support Questionnaire (SSQ) was used to assess social support [32]. The SSQ consists of 2 subscales to quantify the 2 basic elements of social support. The first subscale measures the number of available persons whom the subject feels they can rely on (the number score or SSQ-N). The second subscale assesses the degree of satisfaction with available support (the satisfaction score or SSQ-S). Both scores range from 0 to 36.

36-Item Short Form Health Survey Version 2. The 36-item Short Form Health Survey (SF-36) was developed by Ware Jr. and Sherbourne [33] as a research instrument to evaluate generic health status. It has 8 subscales reflecting physical functioning, physical role, bodily pain, general health perception, vitality, social functioning, emotional role and mental health. Two summary measures, the mental component summary (MCS) and the physical component summary (PCS) scores were further derived from the 8 subscales and were transformed to a mean of 50 and the SD of 10 of the general population according to the manual [27]. Higher scores indicate better health status. A significant predictive value of MCS and PCS for hemodialysis mortality has previously been reported [34].

Follow-Up

The survival status of each subject was confirmed every 6 months for up to 5 years. Information on causes and dates of death was obtained from hospital charts. Subjects who stopped hemodialysis therapy or moved to other clinics were treated as censored cases. During the study period, 11.3% (n = 26) of the subjects were lost to follow-up; 10 patients received transplantations, 5 switched to continuous ambulatory peritoneal dialysis, and 11 changed clinics. We calculated the survival time for each patient as the interval between the date of the first interview and the date of death, the date of censoring, or the end of the study observation, whichever occurred first.

Statistical Analysis

The data were analyzed using SPSS 15.0 for Windows (SPSS Inc., Chicago, Ill., USA). All statistical tests were two-sided. $p < 0.05$ was considered statistically significant, and $p \leq 0.10$ or $p > 0.05$ was considered to be marginal. All values are reported as means ± SD unless otherwise stated.

The baseline characteristics were compared by the presence of depressive symptoms (BDI-II scores of ≥14 or <14) and alexithymia (TAS-20 scores of ≥61 or <61) using the χ^2 statistic test for categorical variables and an unpaired t test for continuous variables.

Kaplan-Meier curves were constructed to demonstrate the association between 5-year mortality according to the presence of depressive symptoms and alexithymia. The Cox proportional hazards regression analysis was used to examine the impact of depression and alexithymia on 5-year mortality risk. The assumption of hazard proportionality was confirmed visually by inspecting the plots of the log cumulative hazards for survival times in patients with and without depression and alexithymia. Depression and alexithymia were treated as continuous variables in the Cox model first, and then the analyses with dichotomized variables were repeated. The presence of a significant interaction between the BDI-II and TAS-20 scores and survival was confirmed by entering the interaction term in the model.

To estimate the respective independent impact of depression and alexithymia on 5-year mortality with adjustment for covariates, multivariate analyses were conducted. Age and sex were added in the Cox model as the primary variables to be adjusted. Then, to exclude the influence of generic health status at baseline, the 2 summary scores of the SF-36 were entered in the model. Finally, baseline variables, of which age and sex adjusted p values of <0.50 in the association with mortality (table 2), were entered in the model and predictors with adjusted $p < 0.50$ retained as covariates in the final model to be adjusted [35].

Results

The mean follow-up period of the 230 patients on chronic hemodialysis was 4.50 ± 1.2 years. In total, 43% (n = 99) of the subjects were depressed according to the BDI-II cutoff score of 14, and 13.9% (n = 32) were alexithymic according to the TAS-20 cutoff score of 61. The major causes of hemodialysis were nephritis (49.1%) and diabetes (26.8%).

There were 27 deaths during the follow-up period. The causes of death were cardiovascular disease (n = 10), infection (n = 5), cerebrovascular disease (n = 3), cancer (n = 2), digestive system disorders (n = 2), external deaths (n = 2) and unspecified (n = 3).

Depression, Alexithymia and Background Characteristics

The background characteristics according to the presence or absence of depression and alexithymia are shown in table 1.

Depressed patients were more likely to be alexithymic (55.2 ± 8.2 vs. 46.0 ± 8.7; $p < 0.001$), to have comorbidity and to have lower social support and health status scores than nondepressed patients. The hematocrit showed a marginally significant difference, whereas other clinical variables were comparable by depression status.

There was no difference between alexithymics and nonalexithymics for any clinical characteristic and sociodemographic variables. In contrast, the alexithymic patients were more depressed (BDI-II score: 21.9 ± 10.4 vs. 12.3 ± 8.4; $p < 0.001$), had less social support and had lower self-report mental and physical health status measures than nonalexithymic patients.

Survival Curves according to Depressive Symptoms and Alexithymia

Figure 1 shows the cumulative survival estimates plotted against time with the presence of depressive symptoms. Depressed patients had a greater mortality risk than nondepressed patients ($p = 0.03$; log rank test).

The survival curves for the alexithymics and nonalexithymics are shown in figure 2. The alexithymic patients showed lower survival curves than the nonalexithymic patients ($p < 0.001$).

Independent Relationship between Depression, Alexithymia and Mortality

Table 2 presents the relationship between the baseline variables and 5-year mortality with adjustment for age and sex. Both of the continuous scores and the dichotomized level of the BDI-II score showed significant associations with an increased risk for 5-year mortality. The mortality risk was increased by 5% per 1-point increase in the BDI-II score, and the depressed patients had a 2.36-fold higher mortality risk compared with the nondepressed patients. The continuous TAS-20 score and its dichotomized level also showed significant associations with an increased risk for 5-year mortality. The mortal-

Table 1. Baseline characteristics of the 230 hemodialysis patients with and without depression and alexithymia

	Total sample (n = 230)	Depressed BDI-II ≥14 (n = 99)	Nondepressed BDI-II <14 (n = 131)	p	Alexithymic TAS-20 ≥61 (n = 32)	Nonalexithymic TAS-20 <61 (n = 198)	p
Sociodemographic characteristics							
Age, years	56.0 ± 9.6	56.2 ± 9.4	55.9 ± 9.7	0.77	55.6 ± 9.8	56.1 ± 9.5	0.76
Female	101 (43.9)	40 (40.4)	61 (46.6)	0.35	12 (37.5)	89 (44.9)	0.43
Married	174 (75.3)	67 (67.7)	107 (81.7)	0.01	22 (68.8)	152 (76.8)	0.33
Education ≥12 years	35 (14.3)	11 (11.1)	24 (18.3)	0.13	5 (15.6)	30 (15.2)	0.95
Clinical characteristics							
Duration of hemodialysis, years	7.28 ± 6.37	7.40 ± 6.50	7.20 ± 6.30	0.84	6.90 ± 7.10	7.35 ± 6.26	0.71
Interdialytic weight gain, % dry weight/day	1.70 ± 0.69	1.8 ± 0.7	1.6 ± 0.7	0.11	1.78 ± 0.96	1.68 ± 0.64	0.57
Having comorbidity	98 (42.6)	55 (55.6)	43 (32.8)	0.001	16 (50.0)	82 (41.4)	0.36
Diabetes	57 (24.8)	30 (30.3)	27 (20.6)	0.09	8 (25.0)	49 (24.7)	0.98
Current smoker	62 (27.2)	30 (30.3)	32 (24.4)	0.32	9 (28.1)	53 (26.8)	0.87
PCR, g/kg/day	0.97 ± 0.17	0.97 ± 0.17	0.97 ± 0.16	0.84	0.96 ± 0.22	0.97 ± 0.16	0.70
Kt/V	1.42 ± 0.22	1.42 ± 0.21	1.43 ± 0.22	0.83	1.37 ± 0.19	1.43 ± 0.22	0.15
Hematocrit, %	32.5 ± 3.3	32.0 ± 3.5	32.8 ± 3.2	0.06	32.2 ± 4.70	32.5 ± 3.07	0.71
Phosphorus, mg/dl	5.41 ± 1.11	5.48 ± 1.12	5.36 ± 1.11	0.40	5.46 ± 1.12	5.40 ± 1.11	0.77
Calcium, mEq/l	4.86 ± 1.15	4.88 ± 1.27	4.85 ± 1.05	0.88	4.84 ± 1.38	4.87 ± 1.11	0.91
Albumin, g/dl	21.5 ± 27.6	19.2 ± 26.5	23.1 ± 28.3	0.29	19.0 ± 27.1	21.9 ± 27.7	0.59
Total cholesterol, mg/dl	168.0 ± 36.2	169.8 ± 39.1	166.6 ± 33.9	0.52	162.3 ± 31.6	168.9 ± 36.9	0.34
Systolic blood pressure, mm Hg	149.1 ± 20.2	151.6 ± 22.8	147.3 ± 18.0	0.13	152.3 ± 24.4	148.6 ± 19.5	0.33
Diastolic blood pressure, mm Hg	85.8 ± 11.8	85.7 ± 12.7	85.9 ± 11.2	0.90	86.7 ± 13.4	85.7 ± 11.6	0.65
SSQ scores							
SSQ-N	18.0 ± 9.6	14.9 ± 8.8	20.3 ± 9.6	<0.001	12.9 ± 8.9	18.8 ± 9.5	0.001
SSQ-S	28.9 ± 5.3	27.2 ± 5.6	30.1 ± 4.7	<0.001	26.5 ± 5.3	29.3 ± 5.2	0.006
Health-related quality of life (SF-36)							
PCS score	41.4 ± 10.9	38.8 ± 10.3	43.4 ± 11.0	0.001	34.8 ± 9.8	42.5 ± 10.7	<0.001
MCS score	49.0 ± 10.2	43.5 ± 9.9	53.2 ± 8.2	<0.001	42.4 ± 9.5	50.1 ± 9.9	<0.001

Values are means ± SD or numbers with percentages in parentheses.

ity risk was increased by 7% per 1-point increase in the TAS-20 score, and those who were alexithymic had a 4.29 times higher mortality risk compared with the nonalexithymics. Hematocrit, diastolic blood pressure and PCS score were also significantly associated with mortality risk.

The results of the multivariate analyses with the continuous BDI-II and TAS-20 scores are presented in table 3. When the continuous BDI-II and TAS-20 scores were entered into the age- and sex-adjusted model simultaneously (model 1), only the TAS-20 score was significantly associated with increased mortality risk. There was no significant interaction between the BDI-II and TAS-20 in relation to mortality (p = 0.25). The associations of the BDI-II and TAS-20 with mortality were unchanged even after adjustment for the baseline health status (model 2). After further adjustment for covariates (education, having comorbidity, interdialytic weight gain, hematocrit, phosphorus, calcium and diastolic blood pressure), the effect of depression on mortality risk was weakened, whereas the increased risk of mortality associated with the TAS-20 score was enlarged from 6 to 7% per 1-point increase (model 3).

The same analytic procedures were repeated with the dichotomized levels of the BDI-II and TAS-20 (table 4). In the final model (model 6), the all-adjusted mortality risk associated with depression was 1.70 (95% CI: 0.64–4.48; p = 0.29), and that with alexithymia was 3.62 (95% CI: 1.32–9.93; p = 0.01).

Discussion

As expected, depression and alexithymia were significantly associated with increased all-cause mortality in hemodialysis patients. However, depression lost its statistical significance after controlling for alexithymia, whereas alexithymia remained significant even after ad-

Table 2. Relationships between baseline variables and 5-year mortality among the 230 hemodialysis patients

	HR adjusted for age and sex	95% CI	p
BDI-II (continuous)	1.05	1.01–1.09	0.01
BDI-II score ≥14	2.36	1.08–5.15	0.03
TAS-20 (continuous)	1.07	1.02–1.11	0.002
TAS-20 score ≥61	4.29	1.95–9.42	<0.001
Age	1.06	1.01–1.12	0.02
Female	0.86	0.39–1.89	0.72
Married	0.49	0.22–1.11	0.09
Education ≥12 years	1.58	0.59–4.27	0.36
Duration of hemodialysis	0.98	0.92–1.04	0.51
Interdialytic weight gain	1.70	0.97–2.98	0.07
Having comorbidity	1.89	0.87–4.11	0.11
Diabetes	1.43	0.64–3.20	0.38
Current smoker	0.78	0.30–2.00	0.60
PCR	1.99	0.21–19.2	0.55
Kt/V	0.86	0.11–6.41	0.88
Hematocrit	0.82	0.73–0.92	0.001
Phosphorus	1.25	0.87–1.80	0.24
Calcium	1.18	0.92–1.52	0.19
Albumin	1.00	0.99–1.02	0.69
Total cholesterol	1.00	0.99–1.01	0.84
Systolic blood pressure	1.00	0.97–1.04	0.86
Diastolic blood pressure	0.97	0.94–1.00	0.03
Perceived social support			
SSQ-N	0.97	0.93–1.01	0.14
SSQ-S	0.98	0.91–1.06	0.63
Health-related QOL (SF-36)			
PCS	0.95	0.92–0.98	0.003
MCS	1.00	0.96–1.04	0.89

All variables were entered individually into the Cox proportional hazard models with age and sex. HR = Hazard ratio; QOL = quality of life.

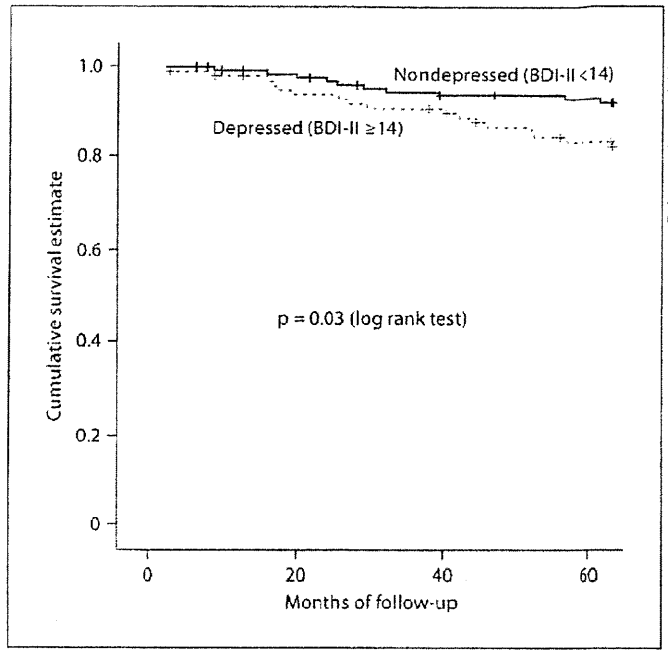

Fig. 1. Kaplan-Meier survival curve for time to death according to the presence of depressive symptoms. All-cause death-free survival in relation to dichotomized level of BDI-II score in hemodialysis patients.

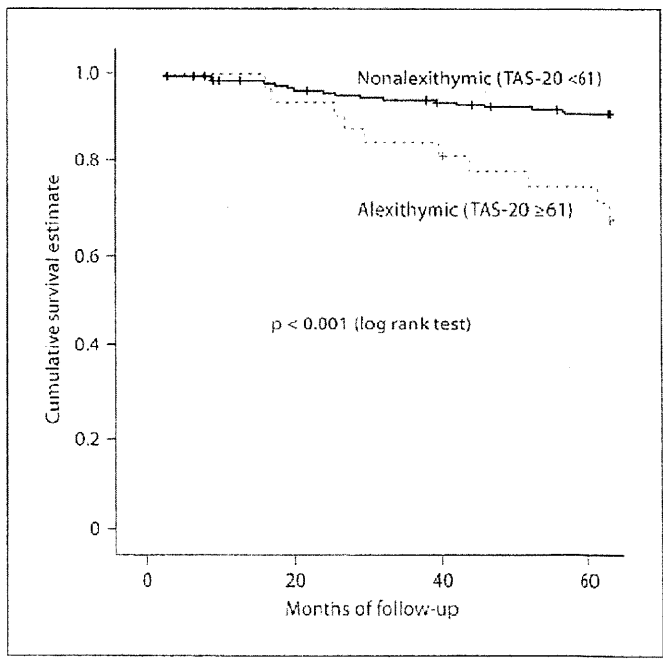

Fig. 2. Kaplan-Meier survival curve for time to death according to the level of alexithymia. All-cause death-free survival in relation to dichotomized level of the TAS-20 score in hemodialysis patients.

justing for depression, current health status and other covariates. To our knowledge, this is the first study reporting an association between alexithymia and long-term mortality in a clinical population.

A number of studies have reported the adverse effect of depression on health outcomes, whereas the evidence suggesting the influence of alexithymia on health is limited. Nevertheless, our findings clearly indicate the superiority of alexithymia to depression as a predictor of long-term mortality in hemodialysis patients.

Theoretically, alexithymia has adverse influences on health outcomes through various pathways [13]. First, alexithymic individuals have difficulties differentiating

Table 3. Multivariate adjusted HR for 5-year mortality among the 230 hemodialyzed patients associated with the continuous scores of the TAS-20 and BDI-II

	TAS-20 score			BDI-II score			Change from previous step		
	HR[1]	95% CI	p	HR[2]	95% CI	p	χ^2	d.f.	p
Model 1: TAS-20, BDI-II, age, sex	1.06	1.01–1.11	0.03	1.02	0.98–1.07	0.37			
Model 2: model 1 + PCS and MCS scores	1.06	1.01–1.12	0.03	1.03	0.98–1.08	0.27	9.00	2	0.01
Model 3: model 2 + covariates[3]	1.07	1.01–1.14	0.03	1.01	0.96–1.07	0.76	17.10	6	0.009

HR = Hazard ratio; d.f. = degree of freedom.

[1] HR shows the increase in mortality risk by 1-point increase in TAS-20 score. [2] HR per 1-point increase in BDI-II score. [3] Variables included in model 3 as covariates were education of ≥12 years, interdialytic weight gain, having comorbidity, hematocrit, calcium and diastolic blood pressure.

Table 4. Multivariate adjusted HR for 5-year mortality among the 230 hemodialyzed patients associated with alexithymia and depression

	Alexithymia, TAS-20 ≥61			Depression, BDI-II ≥14			Change from previous step		
	HR[1]	95% CI	p	HR[2]	95% CI	p	χ^2	d.f.	p
Model 4: alexithymia, depression, age and sex	3.54	1.55–8.11	0.003	1.75	0.77–3.99	0.18			
Model 5: model 4 + PCS and MCS scores	3.64	1.48–8.96	0.005	2.13	0.86–5.23	0.10	7.86	2	0.02
Model 6: model 5 + covariates[3]	3.62	1.32–9.93	0.012	1.70	0.64–4.48	0.29	15.90	6	0.01

HR = Hazard ratio; d.f. = degree of freedom.

[1] HR shows the increased mortality risk associated with the presence of alexithymia (TAS-20 ≥61). [2] HR shows the increased mortality risk associated with the presence of depression (BDI-II ≥14). [3] Variables included in model 6 as covariates were education of ≥12 years, interdialytic weight gain, having comorbidity, hematocrit, calcium and diastolic blood pressure.

and regulating their emotions. They are blunted in recognizing their own physical and emotional symptoms, which may be linked to a delay or excessive use of medical support, resulting in a poor prognosis [36–38]. Second, alexithymic patients tend to amplify and/or misinterpret the somatic sensations that accompany emotional arousal as well as other normal bodily sensations [13]. This may be linked to disturbances of the autonomic and immune systems and the development of somatic diseases [39]. Third, alexithymic patients have a limited ability to cope adaptively with stressful situations, and tend to exhibit unhealthy behaviors such as poor nutrition, alcohol and drug use, and a sedentary lifestyle [13]. Fourth, because of their difficulty communicating their own inner feelings and a poor understanding of other people's emotions, it is hard for alexithymic patients to build and maintain close relationships with others and to utilize social support well [40–42] to protect themselves from the potentially pathological influence of stressful events [43]. However, those pathways just explain an indirect association between alexithymia and premature death. In the present study, alexithymia was not significantly associated with any sociodemographic or clinical characteristics, whereas depression was associated with some baseline variables. It remains unclear why alexithymia has shown a stronger impact on 5-year mortality risk than depression.

There are several studies that have reported the predictive value of alexithymia for health outcomes. Kauhanen et al. [17] examined 2,297 middle-aged Finnish men over a 5.5-year period and found that alexithymia was associated with twice the risk for mortality independently of the effect of well-known behavioral, biological and psychosocial factors. They followed up the study participants for an average of 20 years and recently reported the increased cardiovascular mortality associated with

alexithymia [44]. In accordance with our results, they found no association between alexithymia and biological variables at baseline and no possible mechanisms were addressed. Porcelli et al. [16] examined alexithymia and depression in patients with functional gastrointestinal disorders and compared the improved (n = 68) and unimproved patients (n = 44) after a 6-month treatment. They reported a stronger predictive effect of alexithymia than of depression; however, there was no causal explanation. Further studies are needed to find a clue to clarify the mechanism underlying the association between alexithymia and health outcomes.

According to our recent study, alexithymia was a significant predictor of deterioration in depressive symptoms after 6 months in hemodialysis patients [18]. Because of an inability to self-regulate emotion and a limitation on available support, alexithymic patients are vulnerable to depression under stressful situations and find it difficult to recover [45]. The persistence of depressive symptoms as a strong predictor of mortality in ESRD patients has been suggested by several studies [4, 5]. Combining the previous findings and the present results, the effect of alexithymia on 5-year mortality may partly reflect that of chronic depression which is developed during the follow-up. Because of a limited amount of available data, we could not distinguish the influence of depression developed during the follow-up from that of baseline alexithymia. Alexithymia and depression require different approaches for treatment. Whereas antidepressants and/or psychotherapeutic approaches have been established for the treatment of depression [46], alexithymic patients are generally difficult to be modified [47]. However, an effectiveness of psychotherapy that focuses on increasing affect awareness and imaginative activity has been reported in alexithymic patients [48]. Future studies should disclose the chronological change in depression and alexithymia with and without intervention as well as their independent and joint effects on health outcomes.

The strength of our study is in its prospective design and extensive collection of background characteristics at baseline. This enabled us to estimate the effects of depression and alexithymia on mortality independently of clinical and sociodemographic confounders. To minimize the influences of baseline physical conditions on the present results, we limited the subjects to those who could visit the clinics regularly and complete the self-report questionnaire unaided, and excluded patients who had fatal comorbidity. In comparison with previous studies, our study participants maintained a high survival rate even after the 5-year follow-up. That means our study sample was healthier and more highly compliant than the general hemodialysis patient population. Actually, the prevalence of alexithymia among the present sample was 14%, which is close to the prevalence in the general population. Therefore, to generalize the current findings, further investigations with broader samples are necessary.

There are several limitations to the present study. First, we used self-reporting measures to evaluate the psychosocial characteristics of the patients. Depressed patients tend to have a distorted cognitive style and to perceive themselves negatively [49]. Alexithymic patients also have difficulty perceiving their inner feelings [11]. Therefore, those with severe depression and/or alexithymia may not evaluate their symptoms correctly. It is argued that the psychiatric diagnosis of depression and the depressive symptoms evaluated by self-report measures are not equivalent [1]. According to Hedayati et al. [50], depression diagnosed using a structured physician interview has a stronger prognostic value for ESRD patients than self-reports. Similar results in coronary artery disease patients have also been reported by Frasure-Smith and Lespérance [51]. Considering the findings of these reports, we might have underestimated the influence of depression using a self-report measure, although the BDI has been well validated and used to measure the severity of depression in a number of ESRD patient studies [29]. Our results should be verified using objective measuring tools.

Second, we used the MCS and PCS scores of the SF-36 version 2 based on the factor structure of the US population data as measures of health status. According to the authors of the Japanese manual, the factorial pattern in Japan may differ from that in the USA so that the use of summary scores needs caution [27, 52]. We computed the summary scores based on the US model and the Japanese model and compared the results of the multivariate analysis. As we confirmed the consistency of the findings, we adopted the summary scores based on the US model that had been used internationally [34].

Third, the sample size of the present study was not large enough to control all potential prognostic covariates. Concato et al. [53] has pointed out that a too small ratio of events per variable can affect the accuracy and precision of the results of proportional hazards regression analysis. They conducted a simulation study and suggested the most prudent ratio of events per variable as being 10 [54]. According to the number of deaths we observed (n = 27), the appropriate number of variables is up to 3, but we have included 12 in models 3 and 6. Therefore,

the results of the multivariate analyses should be interpreted with caution.

In conclusion, alexithymia is a strong independent risk factor for all-cause mortality in hemodialysis patients. To disclose the mechanism underlying the association between alexithymia and mortality, further studies with large samples are needed. Prospective studies with careful observation including chronological change in depression and other covariates are important.

Acknowledgments

This study was supported by a Grant-in Aid for Scientific Research (KAKENHI 21590708) from the Japan Society for the Promotion of Science.

We would like to thank Mr. Koji Watanabe for coordinating the study, Mr. Hiroshi Tomizawa, Mr. Atsuhiro Nakata, Mr. Kaoru Kobayashi, Mr. Koichi Ibuki, Ms. Kayo Asada, Ms. Chie Asakura, Ms. Emiko Inoue, Ms. Chiyomi Yamauchi, Ms. Emiko Yamashiro, Ms. Hitomi Kumazawa and the staff members of the Kaikoukai Central Clinic at Nagoya, Anjoh and Hekikai Kyoritsu Clinics in Japan for collecting data. We also thank all of the participants in this study.

References

1 Kimmel PL: Psychosocial factors in dialysis patients. Kidney Int 2001;59:1599–1613.
2 Cohen S, Rodriquez MS: Pathways linking affective disturbances and physical disorders. Health Psychol 1995;14:374–380.
3 Moussavi S, Chatterji S, Verdes E, Tandon A, Patel V, Ustun B: Depression, chronic diseases, and decrements in health: results from the World Health Surveys. Lancet 2007;370:851–858.
4 Boulware LE, Liu Y, Fink NE, Coresh J, Ford DE, Klag MJ, Powe NR: Temporal relation among depression symptoms, cardiovascular disease events, and mortality in end-stage renal disease: contribution of reverse causality. Clin J Am Soc Nephrol 2006;1:496–504.
5 Kimmel PL, Peterson RA, Weihs KL, Simmens SJ, Alleyne S, Cruz I, Veis JH: Multiple measurements of depression predict mortality in a longitudinal study of chronic hemodialysis outpatients. Kidney Int 2000;57:2093–2098.
6 Lopes AA, Bragg J, Young E, Goodkin D, Mapes D, Combe C, Piera L, Held P, Gillespie B, Port FK: Depression as a predictor of mortality and hospitalization among hemodialysis patients in the United States and Europe. Kidney Int 2002;62:199–207.
7 Hedayati SS, Bosworth HB, Briley LP, Sloane RJ, Pieper CF, Kimmel PL, Szczech LA: Death or hospitalization of patients on chronic hemodialysis is associated with a physician-based diagnosis of depression. Kidney Int 2008;74:930–936.
8 Taylor GJ, Bagby RM: New trends in alexithymia research. Psychother Psychosom 2004;73:68–77.
9 Sifneos PE: The prevalence of 'alexithymic' characteristics in psychosomatic patients. Psychother Psychosom 1973;22:255–262.
10 Marty P, M'Uzan M: La pensee operatoire. Rev Fr Psychanal 1963;27:345–356.
11 Taylor GJ: Disorders of Affect Regulation. Alexithymia in Medical and Psychiatric Illness. Cambridge, Cambridge University Press, 2004.
12 Haviland MG, Hendryx MS, Shaw DG, Henry JP: Alexithymia in women and men hospitalized for psychoactive substance dependence. Compr Psychiatry 1994;35:124–128.
13 Lumley MA, Stettner L, Wehmer F: How are alexithymia and physical illness linked? A review and critique of pathways. J Psychosom Res 1996;41:505–518.
14 Parker JD, Taylor GJ, Bagby RM, Acklin MW: Alexithymia in panic disorder and simple phobia: a comparative study. Am J Psychiatry 1993;150:1105–1107.
15 Zeitlin SB, McNally RJ: Alexithymia and anxiety sensitivity in panic disorder and obsessive-compulsive disorder. Am J Psychiatry 1993;150:658–660.
16 Porcelli P, Bagby RM, Taylor GJ, de Carne M, Leandro G, Todarello O: Alexithymia as predictor of treatment outcome in patients with functional gastrointestinal disorders. Psychosom Med 2003;65:911–918.
17 Kauhanen J, Kaplan GA, Cohen RD, Julkunen J, Salonen JT: Alexithymia and risk of death in middle-aged men. J Psychosom Res 1996;41:541–549.
18 Kojima M, Hayano J, Tokudome S, Suzuki S, Ibuki K, Tomizawa H, Nakata A, Seno H, Toriyama T, Kawahara H, Furukawa TA: Independent associations of alexithymia and social support with depression in hemodialysis patients. J Psychosom Res 2007;63:349–356.
19 Lowrie EG, Lew NL: Death risk in hemodialysis patients: the predictive value of commonly measured variables and an evaluation of death rate differences between facilities. Am J Kidney Dis 1990;15:458–482.
20 Owen WF Jr, Lew NL, Liu Y, Lowrie EG, Lazarus JM: The urea reduction ratio and serum albumin concentration as predictors of mortality in patients undergoing hemodialysis. N Engl J Med 1993;329:1001–1006.
21 Hakim RM, Levin N: Malnutrition in hemodialysis patients. Am J Kidney Dis 1993;21:125–137.
22 Held PJ, Port FK, Wolfe RA, Stannard DC, Carroll CE, Daugirdas JT, Bloembergen WE, Greer JW, Hakim RM: The dose of hemodialysis and patient mortality. Kidney Int 1996;50:550–556.
23 Kimmel PL, Varela MP, Peterson RA, Weihs KL, Simmens SJ, Alleyne S, Amarashinge A, Mishkin GJ, Cruz I, Veis JH: Interdialytic weight gain and survival in hemodialysis patients: effects of duration of ESRD and diabetes mellitus. Kidney Int 2000;57:1141–1151.
24 Kojima M, Furukawa TA, Takahashi H, Kawai M, Nagaya T, Tokudome S: Cross-cultural validation of the Beck Depression Inventory-II in Japan. Psychiatry Res 2002;110:291–299.
25 Fukunishi I, Nakagawa T, Nakamura H, Kikuchi M, Takubo M: Is alexithymia a culture-bound construct? Validity and reliability of the Japanese versions of the 20-item Toronto Alexithymia Scale and modified Beth Israel Hospital Psychosomatic Questionnaire. Psychol Rep 1997;80:787–799.
26 Furukawa TA, Harai H, Hirai T, Kitamura T, Takahashi K: Social Support Questionnaire among psychiatric patients with various diagnoses and normal controls. Soc Psychiatry Psychiatr Epidemiol 1999;34:216–222.
27 Fukuhara S, Bito S, Green J, Hsiao A, Kurokawa K: Translation, adaptation, and validation of the SF-36 Health Survey for use in Japan. J Clin Epidemiol 1998;51:1037–1044.
28 Beck AT, Steer RA: Manual for the Beck Depression Inventory-2. San Antonio, Psychological Corporation, 1996.
29 O'Donnell K, Chung JY: The diagnosis of major depression in end-stage renal disease. Psychother Psychosom 1997;66:38–43.
30 Bagby RM, Parker JD, Taylor GJ: The twenty-item Toronto Alexithymia Scale-I: item selection and cross-validation of the factor structure. J Psychosom Res 1994;38:23–32.
31 Bagby RM, Taylor GJ, Parker JD: The twenty-item Toronto Alexithymia Scale II: convergent, discriminant, and concurrent validity. J Psychosom Res 1994;38:33–40.

32 Sarason BR, Levine HM, Basham RB, Sarason IG: Assessing social support: the Social Support Questionnaire. J Pers Soc Psychol 1983;44:127–139.
33 Ware JE Jr, Sherbourne CD: The MOS 36-item Short-Form Health Survey (SF-36). I. Conceptual framework and item selection. Med Care 1992;30:473–483.
34 Knight EL, Ofsthun N, Teng M, Lazarus JM, Curhan GC: The association between mental health, physical function, and hemodialysis mortality. Kidney Int 2003;63:1843–1851.
35 Steyerberg EW, Eijkemans MJ, Harrell FE Jr, Habbema JD: Prognostic modelling with logistic regression analysis: a comparison of selection and estimation methods in small data sets. Stat Med 2000;19:1059–1079.
36 Brown EL, Fukuhara JT, Feiguine RJ: Alexithymic asthmatics: the miscommunication of affective and somatic states. Psychother Psychosom 1981;36:116–121.
37 Kenyon LW, Ketterer MW, Gheorghiade M, Goldstein S: Psychological factors related to prehospital delay during acute myocardial infarction. Circulation 1991;84:1969–1976.
38 Theisen ME, MacNeill SE, Lumley MA, Ketterer MW, Goldberg AD, Borzak S: Psychosocial factors related to unrecognized acute myocardial infarction. Am J Cardiol 1995;75:1211–1213.
39 Mattila AK, Kronholm E, Jula A, Salminen JK, Koivisto AM, Mielonen RL, Joukamaa M: Alexithymia and somatization in general population. Psychosom Med 2008;70:716–722.
40 Lumley MA, Ovies T, Stettner L, Wehmer F, Lakey B: Alexithymia, social support and health problems. J Psychosom Res 1996;41:519–530.
41 Kojima M, Frasure-Smith N, Lespérance F: Alexithymia following myocardial infarction: psychometric properties and correlates of the Toronto Alexithymia Scale. J Psychosom Res 2001;51:487–495.
42 Kojima M, Senda Y, Nagaya T, Tokudome S, Furukawa TA: Alexithymia, depression and social support among Japanese workers. Psychother Psychosom 2003;72:307–314.
43 Cohen S, Wills TA: Stress, social support, and the buffering hypothesis. Psychol Bull 1985;98:310–357.
44 Tolmunen T, Lehto SM, Heliste M, Kurl S, Kauhanen J: Alexithymia is associated with increased cardiovascular mortality in middle-aged Finnish men. Psychosom Med 2010;72:187–191.
45 Ogrodniczuk JS, Piper WE, Joyce AS: Alexithymia as a predictor of residual symptoms in depressed patients who respond to short-term psychotherapy. Am J Psychother 2004;58:150–161.
46 US Preventive Services Task Force: Screening for depression: recommendations and rationale. Ann Intern Med 2002;136:760–764.
47 Taylor GJ: Recent developments in alexithymia theory and research. Can J Psychiatry 2000;45:134–142.
48 Beresnevaite M: Exploring the benefits of group psychotherapy in reducing alexithymia in coronary heart disease patients: a preliminary study. Psychother Psychosom 2000;69:117–122.
49 Beck AT: Depression. Clinical, Experimental, and Theoretical Aspects. New York, Harper and Row, 1967.
50 Hedayati SS, Bosworth HB, Kuchibhatla M, Kimmel PL, Szczech LA: The predictive value of self-report scales compared with physician diagnosis of depression in hemodialysis patients. Kidney Int 2006;69:1662–1668.
51 Frasure-Smith N, Lespérance F: Depression and anxiety as predictors of 2-year cardiac events in patients with stable coronary artery disease. Arch Gen Psychiatry 2008;65:62–71.
52 Fukuhara S, Ware JE, Kosinski M, Wada S, Gandek B: Psychometric and clinical tests of validity of the Japanese SF-36 Health Survey. J Clin Epidemiol 1998;51:1045–1053.
53 Concato J, Peduzzi P, Holford TR, Feinstein AR: Importance of events per independent variable in proportional hazards analysis. I. Background, goals, and general strategy. J Clin Epidemiol 1995;48:1495–1501.
54 Peduzzi P, Concato J, Feinstein AR, Holford TR: Importance of events per independent variable in proportional hazards regression analysis. 2. Accuracy and precision of regression estimates. J Clin Epidemiol 1995;48:1503–1510.

Strong interaction between the effects of alcohol consumption and smoking on oesophageal squamous cell carcinoma among individuals with *ADH1B* and/or *ALDH2* risk alleles

Fumiaki Tanaka,[1] Ken Yamamoto,[2] Sadao Suzuki,[3] Hiroshi Inoue,[1] Masahiko Tsurumaru,[4] Yoshiaki Kajiyama,[4] Hoichi Kato,[5] Hiroyasu Igaki,[5] Koh Furuta,[6] Hiromasa Fujita,[7] Toshiaki Tanaka,[7] Yoichi Tanaka,[8] Yoshiyuki Kawashima,[8] Shoji Natsugoe,[9] Tetsuro Setoyama,[9] Shinkan Tokudome,[3] Koshi Mimori,[1] Naotsugu Haraguchi,[1,10] Hideshi Ishii,[1,10] Masaki Mori[1,10]

► Additional figures and tables are published online only. To view these files please visit the journal online (http://gut.bmj.com).

[1]Department of Surgery, Medical Institute of Bioregulation, Kyushu University, Beppu, Japan
[2]Department of Molecular Genetics, Medical Institute of Bioregulation, Kyushu University, Fukuoka, Japan
[3]Department of Public Health, Nagoya City University Graduate School of Medical Sciences, Nagoya, Japan
[4]Department of Esophageal and Gastroenterological Surgery, Juntendo University School of Medicine, Tokyo, Japan
[5]Department of Surgery, National Cancer Center Hospital, Tokyo, Japan
[6]Division of Clinical Laboratories, National Cancer Center Hospital, Tokyo, Japan
[7]Department of Surgery, Kurume University School of Medicine, Kurume, Japan
[8]Division of Gastroenterological Surgery, Saitama Cancer Center, Saitama, Japan
[9]Department of Surgical Oncology and Digestive Surgery, Kagoshima University School of Medicine, Kagoshima, Japan
[10]Department of Gastroenterological Surgery, Osaka University Graduate School of Medicine, Osaka, Japan

Correspondence to
Dr Masaki Mori, Department of Gastroenterological Surgery, Osaka University Graduate School of Medicine, 2-2 Yamadaoka, Suita, Osaka, Japan 565-0871; mmori@gesurg.med.osaka-u.ac.jp

Revised 5 July 2010
Accepted 8 July 2010
Published Online First 9 September 2010

ABSTRACT

Background Oesophageal squamous cell carcinoma (OSCC) is considered a difficult cancer to cure. The detection of environmental and genetic factors is important for prevention on an individual basis.

Objective To identify groups at high risk for OSCC by simultaneously analysing both genetic and environmental risk factors.

Methods A multistage genome-wide association study of OSCC in Japanese individuals with a total of 1071 cases and 2762 controls was performed.

Results Two associated single-nucleotide polymorphisms (SNPs), as well as smoking and alcohol consumption, were evaluated as genetic and environmental risk factors, respectively, and their interactions were also evaluated. Risk alleles of rs1229984 (*ADH1B*) and rs671 (*ALDH2*) were highly associated with OSCC (odds ratio (OR)=4.08, $p=4.4\times10^{-40}$ and OR=4.13, $p=8.4\times10^{-76}$, respectively). Also, smoking and alcohol consumption were identified as risk factors for OSCC development. By integrating both genetic and environmental risk factors, it was shown that the combination of rs1229984 and rs671 risk alleles with smoking and alcohol consumption was associated with OSCC. Compared with subjects with no more than one environmental or genetic risk factor, the OR reached 146.4 (95% CI 50.5 to 424.5) when both environmental and genetic risk factors were present. Without the genetic risks, alcohol consumption did not correlate with OSCC. In people with one or two genetic risk factors, the combination of alcohol consumption and smoking increased OSCC risk.

Conclusions Analysis of *ADH1B* and *ALDH2* variants is valuable for secondary prevention of OSCC in high-risk patients who smoke and drink alcohol. In this study, SNP genotyping demonstrated that the *ADH1B* and/or *ALDH2* risk alleles had an interaction with smoking and, especially, alcohol consumption. These findings, if replicated in other groups, could demonstrate new pathophysiological pathways for the development of OSCC.

Significance of this study

What is already known about this subject?

► Oesophageal squamous cell carcinoma (OSCC) is associated with drinking and smoking alcohol, but the genetic risk is unknown.

What are the new findings?

► This study demonstrates that single nucleotide polymorphisms of *ADH1B* and *ALDH2* interact with alcohol consumption, especially when combined with smoking, to increase OSCC risk.

How might they impact on clinical practice in the foreseeable future?

► The analysis of *ADH1B* and *ALDH2* variants would be valuable for individualised prevention of OSCC.

INTRODUCTION

Oesophageal squamous cell carcinoma (OSCC), but not adenocarcinoma, is relatively common in East Asia, including Japan.[1] Oesophageal cancer is the eighth most common cancer world wide, accounting for 462 000 new cases in 2002, and the sixth most common cause of cancer-related death (386 000 deaths). OSCC is the most common histological type world wide,[2] and is a treatment-resistant cancer that can withstand a combination of surgery, chemotherapy and radiotherapy.[1] It is difficult to diagnose OSCC early because it shows few symptoms in its early stages. Furthermore, there is no effective marker for predicting the development of OSCC. Therefore, it is important to detect risk factors for primary prevention and also to identify high-risk groups for secondary prevention.

Both genetic and environmental factors are involved in the pathogenesis of OSCC. Although smoking and alcohol consumption have been demonstrated as lifestyle factors that contribute to the development of the disease,[3] the DNA sequence variations that confer an additional risk of developing the disease remain largely unknown. The availability of high-resolution linkage disequilibrium (LD) maps and comprehensive sets of common single nucleotide polymorphisms (SNPs) that capture most of the common sequence

variations facilitate the identification of disease-related genes with genome-wide association studies, an approach without an a priori hypothesis based on a gene function or disease pathway.

To identify OSCC-related genes, we conducted a multistage genome-wide association study in Japanese individuals, with a total of 1071 cases and 2762 controls, and identified a significant genome-wide level of association for two and six SNPs on chromosomes 4q23 and 12q24.11-13, respectively. The most functional variants in the two regions, rs1229984 (*ADH1B*) and rs671 (*ALDH2*),[4] were strongly associated with OSCC. Furthermore, we analysed the association with OSCC of smoking and drinking alcohol, two of the principal environmental determinants of OSCC, both individually and jointly.[5] Finally, we evaluated the combined effects of environmental and genetic risk factors.

METHODS

Study sample

This case–control study was designed to investigate the environmental and genetic risk factors for OSCC. The eligibility criterion was that the oesophageal disease was pathologically diagnosed as OSCC. Patients with newly diagnosed oesophageal cancer, 35–85 years of age, were identified from six hospitals (Juntendo University Hospital, National Cancer Center Hospital, Kurume University Hospital, Saitama Cancer Center, Kagoshima University Hospital and Kyushu University Hospital) from 2000 to 2008. Healthy controls without a previous cancer history were recruited from Kyushu University Hospital (and related hospitals) during the same time period. All controls were enrolled after receiving an upper gastrointestinal endoscopy test to ensure that they had no disease. All participants provided written informed content. The study protocol was reviewed and approved by Kyushu University (Fukuoka, Japan), Juntendo University (Tokyo, Japan), National Cancer Center Hospital (Tokyo, Japan), Kurume University (Kurume, Japan), Saitama Cancer Center (Saitama, Japan) and Kagoshima University (Kagoshima, Japan). In total, 1071 patients with OSCC and 2762 controls were enrolled.

Environmental risk factors

Detailed information about demographic characteristics, lifestyle and daily diet was collected using a standardised questionnaire. Of all the known determinants of OSCC, we chose the two major ones—smoking and alcohol consumption—as environmental risk factors to investigate in detail. Information on smoking and alcohol consumption habits (eg, current smoker, ex-smoker, or non-smoker for smoking status) was collected at the time of enrolment. In addition, the Brinkman index (product of the number of cigarettes per day and years of smoking) for current smokers and years after quitting smoking or drinking (<1 year, 1–2 years, 3–9 years, or 10 years or longer) were calculated. Of the data collected from 1071 patients with OSCC and 2762 controls, the data from 742 patients with OSCC and 820 controls were analysed.

Genotyping, quality control and genetic association analysis

The genome-wide association study was carried out using the Affymetrix GeneChip Human Mapping 500K array (online supplementary figure 1). We genotyped 226 OSCC cases and 1118 controls using the Bayesian Robust Linear Model with Mahalanobis (BRLMM) algorithm. Samples with a genotype call rate <0.94 for either *Nsp*I or *Sty*I GeneChip SNPs were removed from analysis (N=12). To detect duplicated samples, relatives, and DNA-contaminated samples, pairwise identity-by-descent (IBD) estimation was carried out. We detected 1, 28 and 2 pairs showing IBD (PI_HAT) proportions of 1.0, approximately 0.5 and 0.25, respectively. Based on the results, 31 samples that had lower genotype call rates in each pair were excluded from the association analysis. In addition, we removed samples that had deviated averages of PI_HAT (approximately more than 3 standard deviations (PI_HAT > 0.020, N=13, see supplementary figure 2)) because such high mean PI_HAT values might be caused by DNA contamination or low-quality genotyping. These 13 samples also had higher rates of heterozygous genotypes than the other study samples (supplementary figure 3). After the sample quality check, 1288 samples (209 OSCC and 1079 controls) were subjected to further analysis.

SNPs were removed from analysis if they had a call rate of less than 0.95, showed a difference in call rate of more than 0.03 between OSCC and controls, displayed Hardy–Weinberg disequilibrium ($p<1.0\times10^{-4}$) in the control group, or had a minor allele frequency (MAF) <0.10. SNPs that were not selected in the updated GeneChip SNP5.0 (Affymetrix) were also excluded. After these exclusions, 234 830 SNPs remained in the first stage. The genomic inflation factor based on the median χ^2 value was 1.024 in this genome-wide association analysis (supplementary figure 4), implying that there was no systematic increase of false positives owing to population stratification or to any other form of bias. Six SNPs on chromosome 12q24 were strongly associated with the disease, exceeding the genome-wide significance level of $p=1.0\times10^{-7}$ (supplementary figure 5).

In the second stage, 480 OSCC and 864 control samples were genotyped using the Illumina Golden Gate Assay for the best 1536 SNPs (allelic p<0.013). When multiple SNPs displayed strong LD with each other ($r^2>0.8$), the most closely associated SNP was chosen to avoid redundancy during the selection of the 1536 SNPs. The samples with a genotype call rate <0.98 and SNPs with a call rate <0.98, Hardy-Weinberg disequilibrium ($p<1.0\times10^{-4}$) in the controls, or an MAF <0.05 were excluded from the association analysis. After quality control, 479 OSCC, 863 control and 1419 SNP samples remained, and 66 SNPs had an allele test p<0.05 at this stage.

Among the 26 SNPs that showed an allelic p<0.01 in the second stage, 25 could be genotyped with the TaqMan method in 365 OSCC cases and 780 controls in the third stage. The average SNP call rate of these 25 SNPs was 0.998. We identified 10 SNPs with an allelic p<0.05, and eight SNPs reached a significant genome-wide association level ($p<1\times10^{-7}$) in combined samples. The non-synonymous SNPs rs1229984 (*ADH1B*), rs671 (*ALDH2*) and rs16969968 (*CHRNA5*), as well as the synonymous SNP rs1051730 (*CHRNA3*), were also genotyped in all samples in the first through third stages by the TaqMan method.

Statistical analysis

To evaluate genetic and environmental factors, genotype data for the two SNPs (rs1229984 and rs671) and lifestyle data (smoking and alcohol consumption) were available for 742 OSCC cases and 820 controls. Odds ratios (OR) and 95% CIs (95% CIs) were calculated using unconditional logistic regression models, adjusted for sex, age (5-year categories) and study area (Honshu and Kyushu islands).

The environmental factors—that is, history of smoking and alcohol consumption, were re-categorised into two subclasses according to whether subjects had a previous habit of smoking or drinking; this was done to minimise the effect of disease. To evaluate the interaction effect more simply, we chose the

Gut 2010;**59**:1457–1464. doi:10.1136/gut.2009.205724

dominant or recessive model for both SNPs, combining the heterozygous group into either a wild homozygous or mutant homozygous group. The model was selected based on the fitness of the logistic regression. For the results, subjects with GA at rs1229984 were included in the group of AA homozygotes because the recessive model was a better fit than the dominant model. In contrast, the AG and AA genotypes of rs671 were combined because the dominant model was a better fit.

First, we estimated the environmental risk arising from smoking and alcohol consumption both individually and in combination (risk=0, 1 or 2). Similarly, for genetic risk, we estimated the OR of each factor of rs671 (AG/AA) and rs1229984 (GG) and their combined effect (risk=0, 1 or 2). Next, we repeated the same analysis for environmental risk according to the stratum of genetic risk. In the stratified analysis, we evaluated how the environmental effect was modified in the different genetic strata—that is, the existence of a gene–environment interaction. Here we used subjects with the AG/AA genotype of rs671 and/or GG genotype of rs1229984 as a genetic risk group. Finally, we calculated the risk number for the four risk factors in comparison with subjects who had no risk factors to evaluate the accumulation of risk (risk=0, 1, 2, 3 or 4) (tables 1, 2, and 3 and Figure 1).

p Values for the interaction are based on likelihood ratio tests that compared models with and without interaction terms.

Table 1 Characteristics of the cases and controls

Characteristics	Cases (N=742)		Controls (N=820)	
Sex				
Male	641	(86.4)	506	(61.7)
Female	101	(13.6)	314	(38.3)
Age (years)				
40–49	24	(3.2)	127	(15.5)
50–59	149	(20.1)	247	(30.1)
60–69	330	(44.5)	256	(31.2)
70–79	239	(32.2)	190	(23.2)
Environmental risk factor				
Non-drinker	63	(8.5)	341	(41.6)
Ever-drinker (environmental risk)	679	(91.5)	479	(58.4)
Non-smoker	103	(13.9)	422	(51.5)
Ever-smoker (environmental risk)	639	(86.1)	398	(48.5)
Environmental risk No=0	36	(4.9)	252	(30.7)
Environmental risk No=1	94	(12.7)	259	(31.6)
Environmental risk No=2	612	(82.5)	309	(37.7)
Genetic risk factor				
rs671 GG	194	(26.1)	502	(61.2)
rs671 AG/AA (genetic risk)	548	(73.9)	318	(38.8)
rs1229984 AA/AG	591	(79.6)	776	(94.6)
rs1229984 GG (genetic risk)	151	(20.4)	44	(5.4)
Genetic risk No=0	169	(22.8)	479	(58.4)
Genetic risk No=1	447	(60.2)	320	(39.0)
Genetic risk No=2	126	(17.0)	21	(2.6)
Total risk				
Total risk No=0	15	(2.0)	115	(14.0)
Total risk No=1	44	(5.9)	266	(32.4)
Total risk No=2	187	(25.2)	348	(42.4)
Total risk No=3	385	(51.9)	87	(10.6)
Total risk No=4	111	(15.0)	4	(0.5)

Results are shown as number (%).
No: The environmental risk arising from smoking and alcohol consumption, both individually and in combination (risk=0, 1 or 2). Similarly, for the genetic risk of each factor of rs671 (AG/AA) and rs1229984 (GG) and their combined effect (risk=0, 1 or 2). Finally, we calculated the risk number for the four risk factors in comparison with subjects who had no risk factors to evaluate the accumulation of risk (risk=0, 1, 2, 3 or 4).

Table 2 Risk of oesophageal squamous cell carcinoma associated with environmental and genetic risk factors and their internal interaction

Risk factors	Cases	Controls	OR	95% CI
Environmental risk factor				
Non-drinker and non-smoker (environmental risk No=0)	36	252	1.0	Reference
Ever-drinker and non-smoker (environmental risk No=1)	67	170	3.5	(2.1 to 5.8)
Non-drinker and ever-smoker (environmental risk No=1)	27	89	2.3	(1.2 to 4.3)
Ever-drinker and ever-smoker (environmental risk No=2)	612	309	16.0	(9.7 to 26.3)
p Value for interaction of drinking and smoking			0.048	
Genetic risk factor				
rs671 GG and rs1229984 AA/AG (genetic risk No=0)	169	479	1.0	Reference
rs671 AG/AA and rs1229984 AA/AG (genetic risk No=1)	422	297	4.8	(3.7 to 6.3)
rs671 GG and rs1229984 GG (genetic risk No=1)	25	23	3.1	(1.6 to 6.1)
rs671 AG/AA and rs1229984 GG (genetic risk No=2)	126	21	34.0	(18.1 to 63.8)
p Value for interaction of two genetic factors			0.079	

The odds ratios and the 95% CIs for oesophageal squamous cell carcinoma associated with alcohol consumption, smoking, and single nucleotide polymorphisms were estimated from logistic regression models adjusted for sex, age and study area. No: The environmental risk arising from smoking and alcohol consumption, both individually and in combination (risk=0, 1 or 2). Similarly, for the genetic risk of each factor—rs671 (AG/AA) and rs1229984 (GG)—and their combined effect (risk=0, 1 or 2). Finally, we calculated the risk number for the four risk factors in comparison with subjects who had no risk factors to evaluate the accumulation of risk (risk=0, 1, 2, 3 or 4).

Statistical analyses were performed with SAS software version 9.1 (SAS Institute). A two-tailed p value of <0.05 was considered statistically significant.

Genotype data cleaning and IBD analysis were carried out using PLINK version 1.06 software.[6] LD was assessed with HaploView version 4.0.[7] The statistical power for the allelic association analysis in the first and second stages of this study was calculated using the PS program[8] (supplementary table 1). Statistical analyses for the gene–environment interaction were performed with SAS. A two-tailed p value of <0.05 was considered statistically significant.

RESULTS

Figure 2 shows the study design. Table 1 shows several characteristics of the cases and controls. Cases included more men, older individuals, ever-drinkers, ever-smokers and subjects with the AG/AA genotype of rs671 and GG genotype of rs1229984 than controls. The average risk was significantly higher among cases (2.7) than among controls (1.5).

Our multistage association study identified two and six SNPs on chromosomes 4q23 and 12q24.11-13, respectively, which showed genome-wide evidence for association with OSCC ($p<1.0\times10^{-7}$) (table 4). The disease-associated markers of 4q23 spanned the *ADH* gene cluster region, including seven *ADH* family genes: *ADH1A, ADH1B, ADH1C, ADH4, ADH5, ADH6* and *ADH7* (Figure 3). We searched for functional SNPs in these genes in the SNP database and found one validated non-synonymous SNP in exon 3 of *ADH1B*, rs1229984, with an MAF >0.1 in the East Asian population. In addition, 12q24.12 contains the *ALDH2* gene, which is a well-known key enzyme in alcohol metabolism (Figure 4). This gene also possesses a non-synonymous SNP in exon 12, rs671, that affects its enzymatic activity. We assessed the LD between these functional SNPs and

Table 3 The risk of oesophageal squamous cell carcinoma associated with alcohol consumption and smoking status

Risk factors	rs671 GG and rs1229984 AA/AG (genetic risk No =0)		rs671 AG/AA or rs1229984 GG (genetic risk No=1 or 2)		p Value for interaction*
	OR	95% CI	OR	95% CI	
Environmental risk factor					
Non-drinker and non-smoker (environmental risk No=0)	1.0	Reference	1.1	(0.5 to 2.4)	
Ever-drinker and non-smoker (environmental risk No=1)	1.5	(0.7 to 3.3)	12.1	(5.5 to 26.6)	<0.001
Non-drinker and ever-smoker (environmental risk No=1)	4.5	(1.3 to 15.9)	2.4	(1.1 to 5.3)	0.44
Ever-drinker and ever-smoker (environmental risk No=2)	5.0	(2.5 to 10.1)	62.1	(30.3 to 127.4)	<0.001
p Value for interaction of drinking and smoking	0.55		0.048		

The odds ratios and 95% confidence intervals for oesophageal squamous call carcinoma associated with alcohol consumption and smoking were estimated from logistic regression models adjusted for sex, age, mutual habit and study area.
*p Value for interaction between genetic risk and drinking and/or smoking status.

associated SNP markers. We detected moderate LD between rs1229984 and rs1042026 as well as between rs671 and rs11066280 (r^2=0.66 and 0.87, respectively) in control samples (supplementary figure 5). These observations led us to examine the association of rs1229984 and rs671 with OSCC. We found a stronger association between these SNPs and OSCC (allele test OR=1.82, p $=6.2\times10^{-28}$ and OR =1.78, p $=1.0\times10^{-26}$ for rs1229984 and rs671, respectively) than between marker SNPs and OSCC (allele test OR=1.66, p$=1.8\times10^{-16}$ and OR=1.68, p$=2.5\times10^{-21}$ for rs1042026 on 4q23 and rs11066280 on 12q24, respectively), suggesting that rs1229984 and rs671 might be susceptibility variants for OSCC (table 4). Because the other SNP markers with disease associations reside in introns (eg, rs3805322 and rs2074356 reside in the introns of *ADH4* and *C12orf51*, respectively), we cannot exclude the possibility that they have a biological effect on genes from this region. However, other lines of evidence support a possible role for *ADH1B* and *ALDH2* in the pathogenesis of OSCC.

The risk alleles of rs1229984 in *ADH1B* (G) and rs671 in *ALDH2* (A) encode arginine-48 and lysine-504, respectively,

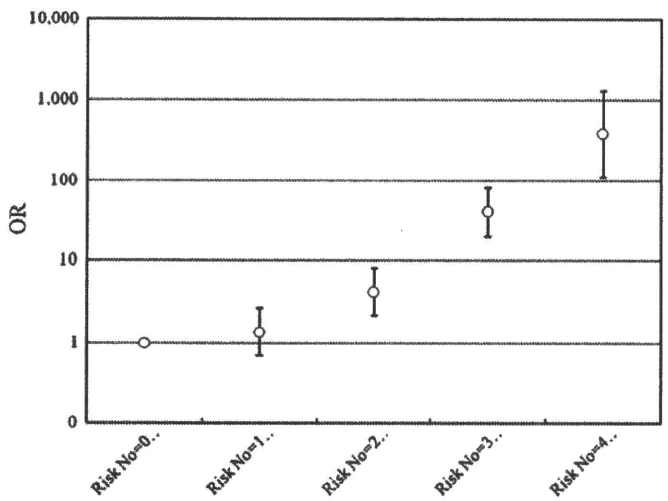

Figure 1 Magnitude of the risk of oesophageal squamous cell carcinoma (OSCC) associated with the number of environmental and genetic risk factors. The odds ratios (ORs) and the 95% CIs for OSCC associated with alcohol consumption, smoking and single nucleotide polymorphisms (rs671 GG and rs1229984) are estimated from logistic regression models adjusted for sex, age and study area. The data are shown as OR+95% CI. No: The environmental risk arising from smoking and alcohol consumption, both individually and in combination (risk=0, 1 or 2). Similarly, for the genetic risk of each factor—rs671 (AG/AA) and rs1229984 (GG)—and their combined effect (risk=0, 1 or 2). Finally, we calculated the risk number for the four risk factors in comparison with subjects who had no risk factors to evaluate the accumulation of risk (risk=0, 1, 2, 3 or 4).

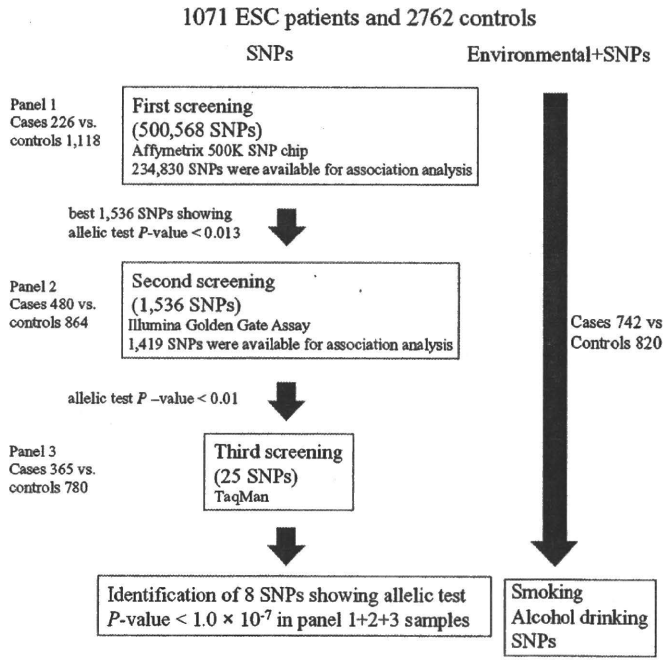

Figure 2 Design of the genome-wide association study and gene-environmental interaction study. In the first stage, 226 patients with oesophageal squamous cell carcinoma (ESC) and 1118 controls were genotyped for 500 568 single nucleotide polymorphisms (SNPs) by Affymetrix 500 K chip sets. Two additional rounds of screening by the Illumina Golden Gate Assay (1536 SNPs for the second screening) and TaqMan Assay (25 SNPs for the third screening) were performed as indicated. To evaluate genetic and environmental factors, genotype data for the two SNPs (rs1229984 and rs671) and lifestyle data (smoking and alcohol consumption) were available for 742 patients with ESC and 820 controls.

Table 4 Association of single nucleotide polymorphisms (SNPs) at 4q23 and 12q24 with oesophageal squamous cell carcinoma (OSCC) in the Japanese samples

SNP (Chromosome; position*)	Screening stage†	Risk allele frequency		OR (95% CI)‡	p Value‡	Genotyping success rate (%)	
		OSCC	Control			OSCC	Control
rs3805322	First	0.45	0.38	1.35 (1.09 to 1.66)	0.0056	100	100
(Chr.4; 100276021)	Second	0.47	0.39	1.42 (1.21 to 1.67)	1.5×10^{-5}	100	100
	Third	0.47	0.35	1.65 (1.38 to 1.97)	4.1×10^{-8}	100	100
	Combined	0.47	0.37	1.48 (1.34 to 1.64)	4.5×10^{-14}	100	100
rs1042026	First	0.22	0.16	1.47 (1.13 to 1.90)	0.0038	100	99.6
(Chr.4; 100447489)	Second	0.26	0.18	1.53 (1.26 to 1.85)	1.0×10^{-5}	99.8	100
	Third	0.26	0.16	1.83 (1.48 to 2.26)	1.9×10^{-8}	99.7	99.9
	Combined	0.25	0.17	1.66 (1.47 to 1.88)	1.8×10^{-16}	99.8	99.8
rs2238149	First	0.30	0.19	1.79 (1.41-2.27)	1.1×10^{-6}	99.0	98.2
(Chr.12; 109796312)	Second	0.26	0.21	1.32 (1.10 to 1.59)	0.0031	100	100
	Third	0.24	0.20	1.26 (1.02 to 1.56)	0.032	100	99.9
	Combined	0.26	0.20	1.41 (1.25 to 1.58)	1.3×10^{-8}	99.8	99.3
rs11065756	First	0.34	0.21	1.92 (1.53 to 2.42)	1.2×10^{-8}	99.5	100
(Chr.12; 109823177)	Second	0.30	0.23	1.43 (1.20 to 1.71)	7.1×10^{-5}	100	100
	Third	0.27	0.22	1.26 (1.03 to 1.54)	0.026	100	99.9
	Combined	0.30	0.22	1.48 (1.33 to 1.66)	7.1×10^{-12}	99.9	100
rs11065783	First	0.40	0.30	1.56 (1.26 to 1.94)	4.9×10^{-5}	100	100
(Chr.12; 109880632)	Second	0.37	0.31	1.35 (1.14 to 1.59)	0.00046	100	100
	Third	0.33	0.28	1.24 (1.02 to 1.50)	0.029	99.7	100
	Combined	0.36	0.29	1.35 (1.21 to 1.50)	3.1×10^{-8}	99.9	100
rs12229654	First	0.34	0.21	1.99 (1.59 to 2.50)	2.1×10^{-9}	99.5	99.8
(Chr.12; 109898844)	Second	0.30	0.21	1.61 (1.35 to 1.93)	1.9×10^{-7}	100	100
	Third	0.27	0.20	1.54 (1.26 to 1.89)	3.4×10^{-5}	99.7	100
	Combined	0.30	0.20	1.66 (1.48 to 1.86)	3.3×10^{-18}	99.8	99.9
rs2074356	First	0.36	0.22	1.97 (1.57 to -2.46)	2.2×10^{-9}	100	100
(Chr.12; 111129784)	Second	0.32	0.23	1.53 (1.29 to 1.83)	1.7×10^{-6}	100	100
	Third	0.32	0.21	1.77 (1.46 to 2.16)	1.1×10^{-8}	100	100
	Combined	0.33	0.22	1.70 (1.52 to 1.90)	3.9×10^{-21}	100	100
rs11066280	First	0.40	0.26	1.92 (1.54 to 2.39)	3.2×10^{-9}	100	99.1
(Chr.12; 111302166)	Second	0.37	0.27	1.52 (1.29 to 1.81)	8.9×10^{-7}	100	100
	Third	0.36	0.25	1.71 (1.41 to 2.06)	2.8×10^{-8}	99.7	99.7
	Combined	0.37	0.26	1.68 (1.51 to 1.87)	2.5×10^{-21}	99.9	99.6

*SNP position is based on NCBI build 36.
†The number of samples at each stage was as follows: 209 and 1079 in the first stage, 479 and 863 in the second stage, and 365 and 780 in the third stage for OSCC and controls, respectively.
‡The odds ratio and p value were calculated by an allele test.

which reduce enzymatic activity (table 5). The frequency of the GG genotype of rs1229984 was higher in OSCC than in controls (0.20 vs. 0.06, OR=4.08, $p=4.4\times10^{-40}$). Similarly, the frequency of the AA+AG genotype of rs671 was higher in cases than in controls (0.73 vs 0.43, OR = 3.54, $p=5.5\times10^{-62}$). These results indicate that individuals who exhibit low enzymatic activity for *ADH1B* and/or *ALDH2* are at higher risk for OSCC.

Table 2 shows the ORs of OSCC associated with environmental and genetic risk factors along with their internal interactions. Ever-drinkers who did not smoke and ever-smokers who did not drink alcohol had significantly elevated adjusted ORs of 3.5 (95% CI 2.1 to 5.8) and 2.3 (95% CI 1.2 to 4.3), respectively. A supra-multiplicative OR of 16.0 (95% CI 9.7 to 26.3)—that is, statistically larger than the product of 3.5 and 2.3 (8.0), was found among individuals who were both ever-drinkers and ever-smokers. Subjects with only one risk allele, either rs671 AG/AA or rs1229984 GG, had significantly higher ORs of 4.8 (95% CI 3.7 to 6.3) and 3.1 (95% CI 1.6 to 6.1), respectively, than those without either risk allele. The OR for those with both genetic risk factors was 34.0 (95% CI 18.1 to 63.8); however, the interaction of these two genetic factors did not reach significance (p=0.079).

We also evaluated the combined effects of environmental and genetic risk factors (table 3). In this analysis, the reference group was composed of individuals who never drank or smoked and who also had no genetic risk factors. Compared with the reference group, ever-drinkers who did not smoke and had no genetic risk factors had a non-significant OR of 1.5 (95% CI 0.7 to 3.3). Non-drinkers and non-smokers with genetic risk factors also had a non-significant OR of 1.1 (95% CI 0.5 to 2.4). All other groups, however, had significantly elevated ORs. An interaction between alcohol drinking and smoking was observed only in the stratum with genetic risk. In the stratum with no genetic risk factors, alcohol drinking was not associated with OSCC, regardless of smoking status. Smoking without alcohol drinking elevated the ORs, regardless of the rs671 and rs1229984 genotypes, similarly and significantly. In contrast, interactions between alcohol drinking and genetic risk factors were highly significant. The combined effects of alcohol drinking and genetic risk factors were larger than the products of individual effects. For example, among non-smokers, the combined OR (12.1) was significantly larger than the product of the genetic effect (1.1) and the alcohol drinking risk (1.5). The same effect was seen among smokers ($62.1>1.1\times5.0$).

Finally, we evaluated the effect on OSCC of the number of risk factors present of the two possible environmental and two possible genetic factors (Figure 1). Compared with the no-risk-factor condition, the ORs for one, two, three and four risk factors were 1.4 (95% CI 0.7 to 2.7), 4.3 (95% CI 2.2 to 8.4), 41.0

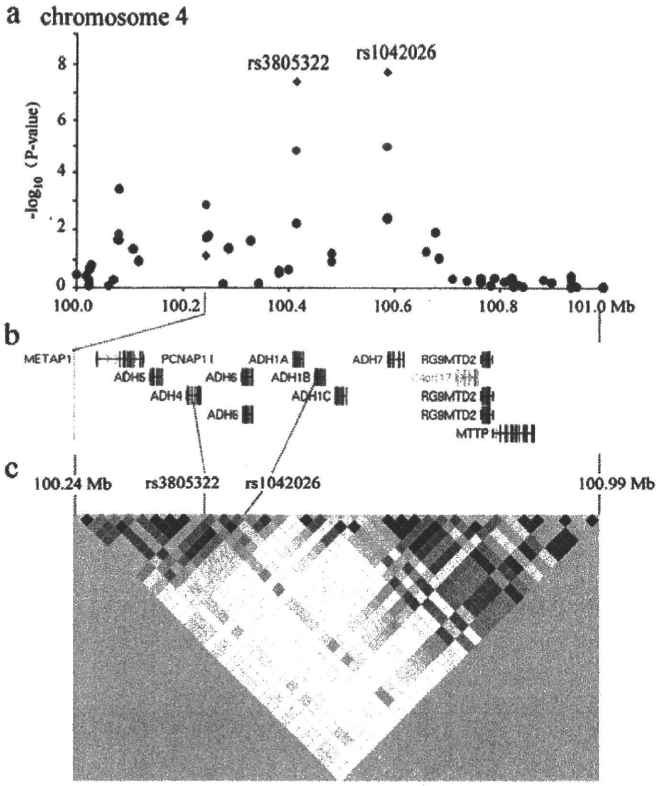

Figure 3 The 4q23 locus is associated with oesophageal squamous cell carcinoma (OSCC). (A) Single nucleotide polymorphism (SNP) single-marker association results. All genotyped SNPs at this locus in this study are plotted with their $-\log_{10}$(p value) for OSCC (allelic test) against chromosome position in Mb (100.0 to 101.0). Black, green and red dots indicate p values at the first, second and third screening stages. Two highly significant SNPs from the combined analysis, rs380532 and rs1042026, are shown. (B) The genomic location of RefSeq genes (100.24–100.99 Mb) with intron and exon structures is shown (UCSC Genome Browser on Human Mar. 2006 Assembly). (C) Pairwise square of the correlation coefficient (r^2) estimates for 39 SNPs from 100.24 Mb to 100.99 Mb in controls at the first stage, with increasing shades of grey indicating higher r^2 values.

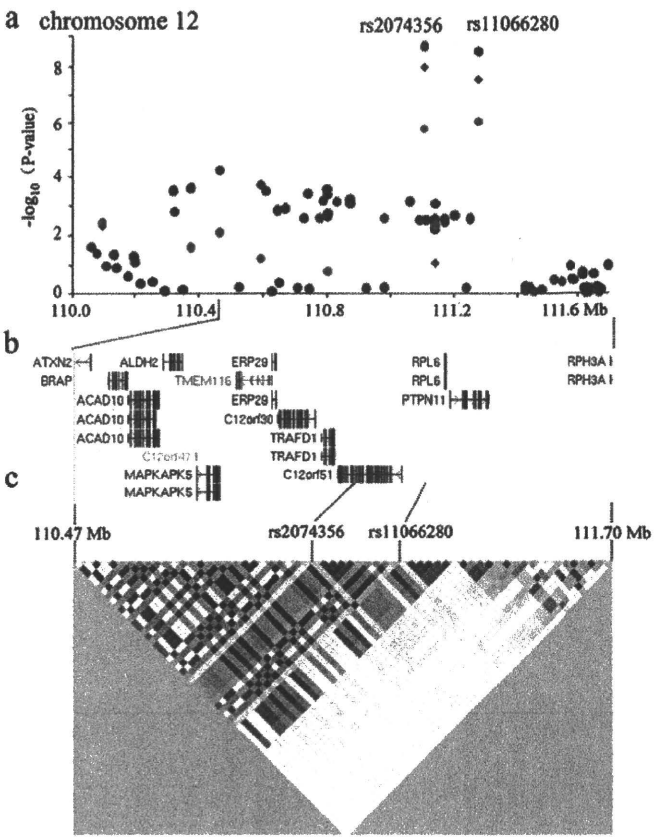

Figure 4 The 12q24.11-13 locus is associated with oesophageal squamous cell carcinoma (OSCC). (A) Single nucleotide polymorphism (SNP) single-marker association results. All SNPs genotyped at this locus in this study are plotted with their $-\log_{10}$(p value) for OSCC (allelic test) against chromosome position in Mb (110.0–111.7). Black, green and red dots indicate p values at the first, second and third screening stages. Two highly significant SNPs in the combined analysis, rs2074356 and rs11066280, are shown. (B) The genomic location of RefSeq genes (110.47–111.70 Mb) with intron and exon structure is shown (UCSC Genome Browser on Human Mar. 2006 Assembly). (C) Pairwise square of the correlation coefficient (r^2) estimates for 63 SNPs from 110.47 Mb to 111.70 Mb in controls at the first stage, with increasing shades of grey indicating higher r^2 values.

(95% CI 20.2 to 83.3) and 357.1 (95% CI 105.4 to 1209.5), respectively. A highly significant linear trend ($p<0.0001$) was observed.

DISCUSSION

Individuals who smoke and drink alcohol are considered at high risk for OSCC, although most such people do not develop the disease. Indeed, in a recent study, only 41 of 100 000 such people developed OSCC.[9] Therefore, it is crucial to simultaneously analyse genetic and environmental risk factors to more efficiently identify people at truly high risk for OSCC. This unbiased genome-wide association study identified two loci

Table 5 Association of non-synonymous single nucleotide polymorphisms (SNPs) in *ADH1B* and *ALDH2* with oesophageal squamous cell carcinoma (OSCC)

SNP (Chromosome; gene)	Study group*	Genotype frequency†			Allele frequency		HWE test p Value	Genotype association test‡ OR (95% CI)	p Value	Allele association test OR (95% CI)	p Value	PAR§ (%)
		GG	GA	AA	G	A						
rs1229984	OSCC	0.20	0.32	0.48	0.36	0.64	9.4×10^{-23}	4.08 (3.27 to 5.09)	4.4×10^{-40}	1.82 (1.63 to 2.03)	6.23×10^{-28}	41.5
(Chr.4; *ADH1B*)	Control	0.06	0.35	0.59	0.23	0.77	0.56					
		AA	AG	GG	A	G						
rs671	OSCC	0.01	0.72	0.27	0.37	0.63	1.7×10^{-81}	3.54 (3.04 to 4.14)	5.5×10^{-62}	1.78 (1.60 to 1.98)	1.03×10^{-26}	38.8
(Chr.12; *ALDH2*)	Control	0.07	0.36	0.57	0.25	0.75	0.12					

The risk allele of rs1229984 (G) and rs671 (A) encode arginine at position 48 and lysine at position 504, respectively, which are known to reduce enzymatic activity.
HWE, Hardy–Weinberg equilibrium.
*The number of OSCC and control samples was 1071 and 2762, respectively.
†The genotyping success rate of rs1229984 and rs671 was 99.84% and 99.79%, respectively.
‡A genotype association analysis was performed for GG versus GA+AA genotype in rs1229984 and for AA+AG versus GG genotype in rs671.
§The population attributable risk (PAR = AF*(OR − 1)/(AF*(OR − 1) + 1)) (AF, allele frequency) is defined as the reduction in the incidence of the disease if the population were not exposed to the risk allele.