Abstract Since the identification of an insertion/deletion (I/D) polymorphism in the angiotensin-converting enzyme (ACE) gene, the D allele has been recognized to be associated with cardiovascular disease. Moreover, significant associations of this polymorphism with multiple cardiovascular risk factors have been reported, although some studies failed to detect such associations. In the present study, we investigated the association of the ACE gene polymorphism with the parameters of multiple risk factors in 300 Japanese men who participated in a medical check-up. This investigation detected a significant association of the polymorphism with systolic blood pressure ($P=0.007$) and diastolic blood pressure ($P=0.026$), with their highest values in DD subjects and lowest values in II subjects. This significant association is consistent with the proposition that the polymorphism influences blood-pressure variability in men. Furthermore, we investigated the association of the polymorphism with four major disorders (obesity, hyperlipidemia, hypertension, diabetes mellitus) correlated with the risk for cardiovascular disease in the same 300 subjects. This investigation failed to detect any significant association of the polymorphism with each disorder. However, there was a trend that all four disorders were more frequent in ID and DD subjects than in II subjects. We therefore analyzed the association between the ACE gene polymorphism and having at least one of the four disorders in the same population. This analysis detected a significant difference: that ID and DD subjects had at least one of the four disorders more frequently than II subjects ($P=0.008$; odds ratio=1.89, 95% confidence interval=1.19–2.99).

Taken together, the results of this study are compatible with the proposition that the ACE polymorphism is associated with cardiovascular disease partially mediated through the four disorders in our population.

Introduction Cardiovascular disease is a multifactorial disease, influenced by environmental and genetic factors. As one of such genetic factors, the deletion allele of the insertion/deletion (I/D) polymorphism in the angiotensin I-converting enzyme (ACE) gene has been shown to be associated with cardiovascular disease (Cambien et al. 1992; Samani et al. 1996). However, the underlying mechanisms of this association are not fully understood. In this context, several studies have detected significant associations of the polymorphism with multiple cardiovascular risk factors (Zingone et al. 1994; Suzuki et al. 1996; Mastana and Nunn 1997; Nakano et al. 1998), although some studies failed to detect such associations. Thus, whether the D allele is associated with cardiovascular disease mediated through cardiovascular risk factors remains inconclusive.

Here, we had the opportunity to test the association of the ACE I/D polymorphism with the parameters of several risk factors and four major disorders (obesity, hyperlipidemia, hypertension, diabetes mellitus) correlated with the risk for cardiovascular disease in 300 Japanese men who participated in a medical check-up. We determined the genotypes (DD, ID, or II) in all the subjects and compared the values of the parameters of the risk factors with the frequencies of the disorders between the three genotypes. Moreover, we compared the frequency of having at least one of the four disorders between the different genotypes.

Materials and methods Subjects

We studied 300 Japanese men who participated in a medical check-up. They had been free from any medication for at least 4 weeks before the study. Their ages ranged from 29 to 59 years...
old. All subjects gave written informed consent before participating in the study, which was approved by the ethics committee of Ehime University.

The study participants were examined after an overnight fast. Weight and height were recorded, and the body mass index (BMI) was calculated (kg/m²). Obesity was defined as BMI ≥26 kg/m². Blood pressure was measured as recommended by the American Heart Association (Kirkendall et al. 1980). The subjects lay supine for 10 min, whereafter the blood pressure was measured with a mercury sphygmomanometer. Special care was taken to control and avoid stimuli that may influence blood pressure. Hypertension was defined as blood pressure ≥140/90 mmHg. Serum total cholesterol, triglyceride (TG), and high-density-lipoprotein (HDL) cholesterol were measured with a standard protocol, and low-density-lipoprotein (LDL) cholesterol level was calculated by the Friedewald equation [LDL cholesterol = total cholesterol – (TG/5) – HDL cholesterol]. Hyperlipidemia was defined as total cholesterol ≥220 mg/dl and/or triglyceride ≥150 mg/dl. Most of the subjects with hyperlipidemia had hypertriglyceridemia. All subjects also underwent a 75-g oral glucose tolerance test, and plasma glucose concentration was measured after 0, 60, and 120 min. Diabetic subjects were identified according to the American Diabetes Association criteria for diabetes mellitus. The subjects were considered to have diabetes mellitus if plasma glucose concentration was ≥126 mg/dl.

Determination of ACE genotypes

DNA was extracted from 2 ml whole blood with a QIAamp Blood Kit (QIAGEN). The polymerase chain reaction (PCR) was used to detect the ACE gene I/D polymorphism (Lindpaintner et al. 1995). The sense oligonucleotide primer was 5'-GCC CTG CAG GTG TCT GCA GCA TGT-3' and the antisense primer was 5'-GGA TGG CTC TCC CCG CCT TGT TCT C-3'. The PCR mixture contained 50 ng genomic DNA, 10 pmol of each primer, 250 µmol/l dNTP, 1.5 mmol/l MgCl₂, 50 mmol/l KCl, 10 mmol/l Tris-HCl, pH 8.4, and 1 U Taq DNA polymerase (Takara Shuzo) in a final volume of 25 µl. The amplification cycle was performed on a PC-801 thermal cycler (ASTEC). After initial denaturation at 94°C for 3 min, the DNA was amplified by 35 PCR cycles: denaturation at 94°C for 30 s, annealing at 58°C for 45 s, and extension at 72°C for 2 min, followed by final extension at 72°C for 7 min. Amplified products were separated by electrophoresis on 2% agarose gel and visualized under ultraviolet light after ethidium-bromide staining. The person who assessed the genotype was blinded to the clinical data of the subject from whom the sample originated. Because the D allele in heterozygous samples is preferentially amplified, each sample with the DD genotype was subjected to an independent PCR amplification with a primer pair that recognizes an insertion-specific sequence (Lindpaintner et al. 1995).

Statistical analysis

Data are presented as mean±SD. The levels of the variables were compared in relation to the three genotypes using analysis of variance (ANOVA). P values less than 0.05 were considered statistically significant.

### Results

Frequencies of alleles and genotypes

The relative frequencies of the II, ID, and DD genotypes were 44, 42, and 14%, respectively. The allele frequencies were 65 and 35% for the I and D alleles, respectively. These results are consistent with the Hardy-Weinberg equilibrium.

Association between genotypes and phenotypes

Table 1 presents the actual values of the parameters associated with risk for cardiovascular disease as a function of the three genotypes. Based on the known association of the ACE-gene I/D polymorphism with cardiovascular disease, we analyzed the differences in the values between the different genotypes. These analyses showed no significant differences in these values, except in systolic blood pressure and diastolic blood pressure. Systolic blood pressure (P=0.007) and diastolic blood pressure (P=0.026) were highest in DD subjects, intermediate in ID subjects, and lowest in II subjects. The variance in blood-pressure distribution appeared attributable to higher blood-pressure levels in the ID and DD subjects (P=0.02 for systolic blood pressure and P=0.01 for diastolic blood pressure; data not shown).

Next, we studied the association of the ACE I/D polymorphism with four major disorders (obesity, hyperlipidemia, hypertension, diabetes mellitus) associated with risk for cardiovascular disease. This resulted in a failure to detect any statistically significant association between the different genotypes (Table 2). However, comparison of the frequency of having at least one of the four factors between the three genotypes showed a significant difference, with the highest frequency in DD subjects, intermediate frequency in ID subjects, and lowest frequency in II subjects (P=0.012). The difference in this distribution appeared attributable to a higher frequency of having at least one of the four factors in the ID and DD subjects.