

## Pro12Ala polymorphism in the PPARG gene contributes to the development of diabetic nephropathy in Chinese type 2 diabetes

*Running title:* PPARG - Pro12Ala and diabetic nephropathy

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*Objective*— Oxidative stress is a major contributing factor in the development of diabetic nephropathy. Peroxisome proliferator-activated receptor gamma (PPAR- $\gamma$ ) heterozygous mice and Pro12Ala polymorphism in PPARG exhibited increased resistance to oxidative stress. Smoking increases the production of reactive oxygen species, which accelerates oxidative stress under hyperglycemia. To determine whether the Pro12Ala polymorphism, alone or in combination with smoking, contributes to the development of diabetic nephropathy, a case-control study was performed in 760 Chinese patients with type 2 diabetes.

*Research design and methods*— Among patients, 532 had diabetic nephropathy with micro-albuminuria (n=245) or overt-albuminuria (n=287), and 228 did not show either of these symptoms but had been suffering from diabetes for  $\geq 10$  years and were not undergoing antihypertensive treatment.

*Results*— After adjusting for confounders, the Pro/Pro genotype significantly associated with diabetic nephropathy, odds ratio (OR) 2.30 (95% CI 1.18-4.45),  $p=0.014$ ; smoking was also an independent risk factor for diabetic nephropathy with OR 1.99 (1.08-3.68),  $p=0.029$ . In addition, we identified possible synergistic effects, i.e., the high-risk group (smokers with the Pro/Pro genotype) showed 4.52 times higher risk (95% CI, 1.78-11.48;  $p=0.002$ ) of diabetic nephropathy than the low-risk group (nonsmokers with Pro/Ala genotype) in a multiple-logistic-regression analysis controlled for the confounders.

*Conclusions*— Our results indicated that the Pro/Pro genotype and smoking were significant independent risk factors for diabetic nephropathy. The possible synergistic effects of genotype and smoking may aggravate oxidative stress and contribute to the development of diabetic nephropathy.

**D**iabetic nephropathy (DN) is a leading cause of end-stage renal disease (ESRD) in developed countries. A recent epidemiological study indicated that albuminuria was present in 49.6% of the Chinese type 2 diabetic patients aged over 30 and living in Shanghai urban area (1). Although the causes of DN have not been fully understood, the familial aggregation of the disease and the disproportionate prevalence among specific ethnic-minority groups suggest that genetic factors may influence the risk of developing the disease (2). The PPARG gene located on chromosome 3, which has been linked to the risk of developing DN. The peroxisome proliferator-activated receptor (PPAR)- $\gamma$ 2 is a transcription factor formed by an alternative mRNA splicing pathway, and it regulates the transcription and expression of numerous target genes, which have been shown to be involved in adipocyte differentiation, lipid and glucose metabolism, and atherosclerosis (3). The Pro12Ala polymorphism, a Pro-to-Ala exchange that results in the substitution of proline with alanine at codon 12, was associated with reductions in both DNA binding and transcriptional activity in vitro, and the Ala12 carriers showed significant improvement in insulin sensitivity (4). This beneficial effect of lower PPAR- $\gamma$  activity on insulin sensitivity in humans can be replicated in PPAR- $\gamma$  heterozygous (PPAR- $\gamma^{+/-}$ ) mice (5). The Pro12Ala polymorphism has also been associated with DN in Caucasians (6-8). Recent studies have indicated that the Ala12-allele-mediated improvement in insulin sensitivity may involve enhanced suppression of lipid oxidation, which permits more efficient glucose disposal (9). Moreover, adipose-tissue-specific PPAR- $\gamma$ 2-heterozygous mice and human Ala12 allele carriers show increased resistance to oxidative stress (9,10). Oxidative

stress resulting from overproduction of reactive oxidant species (ROS) under hyperglycemic conditions has been suggested to contribute to the development and progression of DN (11). Smoking increases the production of ROS (12), consequently accelerating oxidative stress under hyperglycemic conditions. Smoking is also known to increase urinary albumin excretion and is predicted to lead to faster progression of nephropathy in patients with type 2 diabetes (13). A number of epidemiological studies have provided evidence that interactions between genetic and nongenetic risk determinants contribute to the development and progression of DN (14). However, previous studies have shown that the prevalence of Pro12Ala greatly varies among populations; the prevalence in Asians is much lower than that in Caucasians (15). In addition, the Pro12Ala polymorphism was found to be associated with type 2 diabetes in Caucasians, but not in the Chinese (16-17). In this study, we investigated the influence of the PPARG-Pro12Ala polymorphism on the risk of DN and determined whether this polymorphism and smoking showed a synergistic effect on the development of type 2 diabetic nephropathy in Chinese patients.

## RESEARCH DESIGN AND METHODS

### Patient selection and clinical investigation.

We selected the type 2 diabetic subjects (n=760) of Chinese Han ethnicity who were inpatients at the Department of Endocrinology and Metabolism and the Department of Nephrology at Shanghai Jiaotong University Affiliated Sixth People's Hospital between January 2005 and October 2008. Subjects with type 2 diabetes were diagnosed according to the 2003 American Diabetes Association (ADA) diagnostic criteria for diabetes and were divided into no-DN and DN groups according to their 24-h albumin-excretion rates (AER). The no-DN

group (n=228) consisted of patients who had suffered from type 2 diabetes for at least 10 years, were not receiving antihypertensive treatment, and did not show albuminuria (AER < 30 mg/24 h). Ruling out urinary tract infection, hematuria, and nephritis, etc.(18), the DN group was further subdivided into a micro-albuminuria group (n=245, 300 mg/24 h > AER ≥ 30 mg/24 h), and an overt-albuminuria group (n=287, AER ≥ 300 mg/24 h) in at least 2 consecutive overnight samples collected over a 3-to 6-month period. Diabetic retinopathy (DR) was evaluated for all patients simultaneously by an experienced ophthalmologist. In the DN micro-albuminuria group, all the patients were confirmed with co-existence of DR.

All the patients underwent a standardized clinical and laboratory evaluation. Homeostasis model assessment of insulin resistance (HOMA-IR) and HOMA β were calculated by using the formulae described by Matthews et al (19). Information on smoking habits was obtained using questionnaires. Patients who had smoked at least 1 cigarette per day for at least 1 year at the time of study recruitment were stratified into the smoking group, and patients who did not smoke or had given up smoking for > 1 year at the time of study recruitment were stratified into the nonsmoking group. This study was approved by the Institutional Review Board of Shanghai Jiaotong University Affiliated Sixth People's Hospital. Written informed consent was obtained from all the participants.

**Genotyping.** Genomic DNA was extracted from 5 ml of peripheral blood by using the conventional phenol/chloroform method. Mismatch polymerase chain reaction (PCR) and restriction -fragment length polymorphism (RFLP) for *Hae III* digestion were used for genotyping analysis of Pro12Ala polymorphism by using previously established procedures (20). For the genotyping analysis, PCR products with a length of 155 bp were digested to 2 fragments

with sizes of 132 bp and 23 bp, which were for the Pro12 allele, and the complete 155-bp fragment was for the Ala12 allele. To confirm that detection of this C→G nucleotide substitution which resulted in Pro12Ala (CCA→GCA) by PCR-RFLP analysis is reproducible, we performed PCR-based direct sequencing analysis for each study subject.

**Statistical analysis.** Since there were no differences between the genotype frequencies of the micro-albuminuria- and overt-albuminuria groups, we combined these 2 groups into a DN group for further data analyses. The clinical and laboratory values were expressed as means ± S.D. or as median with interquartile range. Comparisons of the clinical and laboratory parameters between the diabetes with and without DN groups as well as those between the genotypic groups were performed with unpaired Student's *t* test and Chi-square analysis, as appropriate. Skewed distribution data such as those for the duration of diabetes, fasting plasma insulin level, HOMA-β, HOMA-IR, triglyceride, AER and serum creatinine (Scr) levels were logarithmically transformed before analysis and are presented as median with interquartile range. P values < 0.05 were considered to be significant. To evaluate the independent contributions of the Pro12Ala polymorphism and smoking to the risk of DN, we performed multivariate-logistic-regression analysis of type 2 diabetic patients with DN (micro- and overt-albuminuria grouped together) by using type 2 diabetic patients without DN as the controls; the analyses included possible confounders of sex, age at diagnosis of diabetes, diabetes duration, hypertension, triglyceride, total cholesterol and HbA1c levels. The odds ratios (ORs) and 95% confidence intervals (CI) were calculated. The statistical package for software sciences (SPSS) 10.0 was used for data analysis and processing.

We used SAS 9.1.3 software (SAS Institute inc., Cary, NC, USA) to calculate sample size

and power. Results of the pilot study which enrolled 94 no-DN and 165 DN patients on association between Pro12Ala and DN showed that the OR of high risk genotype, Pro/Pro to low risk genotype, Pro/Ala was 2.21, we decided to use OR of 2.0 to calculate sample size. Other assumptions used to calculate sample size were that (i) the prevalence of high risk genotype of PPARG was 85%, (ii) the sample size proportion between no-DN group and DN group was 1:2 and (iii) the power was 80% ( $\alpha=0.05$ ), the total sample size needed was 732. In our study, 760 patients including 228 no-DN and 532 DN patients were enrolled. Using current sample, the power to find OR of 2.2 was 82.2% ( $\alpha=0.05$ ), therefore, the sample size was considered to be adequate.

To investigate the possible combined effects of the genotype and smoking on the risk of DN, we used multivariate -logistic-regression analysis with stratification on the basis of genotype (Pro/Pro or Pro/Ala) and smoking status (smokers or nonsmokers). A low-risk genotype and no history of smoking were considered as reference groups. If the combined effect of the 2 factors is 0, then there is no sign of interaction between the factors; consequently, there is no departure from additivity. We used the following formula to calculate the combined effect:  $1 + OR_{A+B+} - OR_{A+B-} - OR_{A-B+}$ , where 1 is the effect of the reference (21).

## RESULTS

In comparison with the no-DN patients, the DN patients shows significant differences in several clinical and laboratory characteristics in this study (Table 1). The crude analysis revealed that the risk of suffering from DN was significantly increased by smoking (OR, 2.18; 95% CI, 1.30-3.68;  $p=0.003$ ).

As shown in Table 2, with the exception of the Pro/Ala patients that had lower AER level than Pro/Pro patients ( $p = 0.001$ ), there were no differences between the Ala12 carriers and

noncarriers with regard to clinical and laboratory characteristics.

All Ala12 carriers were heterozygotes (Pro12Ala); there were no Ala/Ala homozygotes in the population sample studied. The genotypic distribution of the Pro12Ala polymorphism in each group was in Hardy-Weinberg equilibrium ( $P>0.05$ , Figure 1). However, since there were no significant differences between the genotype distributions in the micro- and overt-albuminuria groups, we combined these samples into a DN group, as described in RESEARCH DESIGN AND METHODS (Figure 1). The Pro/Ala genotype and the Ala12-allele frequency in the DN group were clearly lower than those in the non-DN group (6.2% vs. 12.7% for the Pro/Ala genotype, and 3.1% vs. 6.4% for the Ala allele;  $p=0.003$  for both of the parameters; Figure 1).

When the risks of micro- and overt-albuminuria nephropathy were separately analyzed using multivariate-logistic-regression analysis with adjustment for the possible confounders, the Pro/Pro genotype was significantly associated with overt-albuminuria nephropathy, 2.76 (1.22-6.19),  $p=0.014$ . There was a tendency for association with micro-albuminuria nephropathy, that was not statistically significant, 1.94 (0.90-4.14),  $p=0.089$ . In unadjusted analyses, the Pro/Pro genotype was significantly associated with DN, 2.20 (1.30-3.73),  $p=0.003$  (Figure 1).

To evaluate the independent contributions of the polymorphism and smoking to the risk of DN, multivariate-logistic-regression analyses of type 2 diabetic patients with and without DN were performed with the possible confounders. We obtained the following values for the individual confounders: sex, OR, 0.92 (0.62-1.34,  $p=0.649$ ); age at diagnosis of diabetes, OR, 1.02 (1.00-1.04,  $p=0.021$ ); diabetes duration, OR, 0.91 (0.88-0.94,  $p<0.001$ ); hypertension, OR, 2.34 (1.56-3.49,  $p<0.001$ ); triglyceride, OR, 1.59

(1.18-2.14,  $p = 0.002$ ); total cholesterol, OR, 1.10 (0.94-1.29,  $p = 0.222$ ); HbA1c, OR, 0.97 (0.92-1.03,  $p = 0.281$ ) and smoking, OR, 1.99 (1.08-3.68;  $p = 0.029$ ); the Pro/Pro genotype significantly increased the risk of DN: OR, 2.30 (1.18-4.45;  $p = 0.014$ ).

To study the possible interaction between the polymorphism and smoking, we used a variable that stratified the participants according to the genotype and smoking status in a multivariate -logistic-regression analysis that was adjusted for the confounders. The high-risk group (smokers with the Pro/Pro genotype) had a 4.52 times higher risk of DN (95% CI, 1.78-11.48) than the low-risk group (nonsmokers with the Pro/Ala genotype) ( $p = 0.002$ ), and the departure from additivity was 1.40, indicating a possible synergistic interaction between genotype and smoking in patients with DN (Figure 2). Except for the synergistic interaction between smoking and genotype, no synergistic effects were found between smoking and other covariates which were associated with DN.

## DISCUSSION

In our study, the Pro/Pro genotype showed significant risk associations with DN, when adjustments were made for other risk factors. The association with Pro/Pro genotype seemed stronger in overt-albuminuria nephropathy than in micro- albuminuria nephropathy. Despite a larger sample, subdividing the cases into micro- and overt-diabetic nephropathy will lead to a greater risk of statistical instability, but we can't exclude that what we see is an association to overt-diabetic nephropathy and that other factors, stronger than the polymorphism, are more important for the progression from no diabetic nephropathy to micro-albuminuria nephropathy. These results for Chinese patients are in agreement with the findings for Caucasian patients, which were reported by Herrmann et al (6), Caramori et al (7), and Pollex et al (8), who found that the

frequencies of the Ala12 allele in German, Brazilian, and Oji-Cree type 2 diabetic patients with DN were lower than the corresponding frequencies in patients without DN.

The Pro12 allele is more common in the Chinese population than in Caucasian populations (95% vs. 88%) (17, 22), and we observed a stronger significant association with DN. However, the observed stronger genetic association with DN confirms the earlier findings, and this association can be observed across ethnicities, which highlights the importance of this polymorphism in the development of DN. However, previous studies reported that the Ala12 allele was resistant to type 2 diabetes in Caucasians, but not in Chinese, thus reflecting an ethnic genetic heterogeneity of type 2 diabetes at this locus (16,17).

While smoking was also an independent risk factor for the development of DN in the crude analyses as well as the analyses performed after adjustment for possible confounders, smoking was a weaker risk factor for micro-albuminuria nephropathy and a stronger risk factor for macroalbuminuria nephropathy (For the former, OR, 1.70; 95% CI, 0.863-3.351;  $p = 0.125$ ; (For the latter, OR, 2.36; 95% CI, 1.21-4.59;  $p = 0.012$ ), supporting the oxidative -stress hypothesis (11).

In the present study, we observed that both homozygosity for the Pro12 allele of the PPARG-Pro12Ala polymorphism and smoking were associated with a significant increase in the risk of DN, even after adjustments for possible confounders. Moreover, we also detected a possible synergistic effect of these 2 factors on DN, i.e., the high-risk group (smokers with the Pro/Pro genotype) showed 4.52 times higher risk of DN than the low-risk group (nonsmoking patients with the Pro/Ala genotype). The adipose-tissue-specific PPAR- $\gamma$ 2 -Ala12 carriers exhibit increased resistance to

oxidative-stress (9), and smoking increases the production of ROS (12). Thus, the aggravation of oxidative stress under hyperglycemic conditions may reflect the possible synergistic effects of the combination of the Pro/Pro genotype and constant smoking on the development of DN.

The present case-control study was performed using a relatively larger sample size than previous studies and was statistically well powered involving 2 carefully characterized groups of type 2 diabetic patients with and without DN. We identified 2 independent risk factors, i.e., Pro/Pro genotype and smoking, and we found that these 2 risk factors showed synergistic effects on the development of DN in Chinese type 2 diabetic patients, supporting the idea that an interplay between genetic and nongenetic risk determinants contributes to the development and progression of DN (14). The interaction between the genetic risk factor - the PPARG gene and the lifestyle-related risk factor - smoking is an interesting finding; however, these are preliminary findings, and they should be confirmed by studies in other ethnic groups. This is the first China- or Asia-specific report on the effects of the higher prevalence of the PPARG-Pro/Pro genotype and the interaction between this genotype and smoking in the development of DN in type 2 diabetic patients. A large population study in Japanese type 2 diabetic patients, which showed that the prevalence of this genotype in the Japanese patients was similar to that observed in Chinese patients, did not show any effects of the genotype on DN; however, the results of that study were influenced by the standard used for defining DN, i.e., AER>10  $\mu\text{g}/\text{ml}$  (23).

The mechanisms by which the PPARG-Pro12 Ala polymorphism contributes to DN have still not been clarified. Previous studies have suggested that Ala12 carriers show significant improvement in insulin sensitivity (4); this suggestion implies that non-Ala carriers, i.e.,

patients who are homozygous for the Pro12 allele, show increased insulin resistance, which contributes to the development of DN. However, we could not detect the association between the Ala12 allele and the insulin-resistance-related clinical parameters; this finding is consistent with the results of the study by Li et al on diabetic Chinese Hans (17). In addition, the latest studies indicated that mice that were heterozygous for the adipose-tissue-specific PPAR- $\gamma$ 2 and human carriers of the Ala12 allele showed increased resistance to oxidative stress (9, 10). The enhanced oxidative-stress tolerance is associated with significant upregulation of antioxidant genes and a significant increase in the adipose tissue of Foxo3a, a transcription factor that is known to regulate the clearance of ROS (10). The enhanced oxidative-stress tolerance of the Ala12 carrier also implies that the Pro/Pro genotype increases the production of ROS, which accelerates oxidative stress, causing an increase in glomerular albumin permeability, and the degree of proteinuria correlates with the progression of glomerulosclerosis and tubulointerstitial fibrosis (24). Oxidative stress can cause insulin resistance, which is a consequence as well as a potential cause of DN (25).

In conclusion, our results indicated that smoking and the Pro/Pro genotype of Pro12Ala polymorphism in the PPARG gene were significant independent risk factors for DN. The possible synergistic effects between the genotype and smoking implied that positive interaction between genetic and nongenetic factors may aggravate oxidative stress and contribute to the development of DN in Chinese type 2 diabetic patients.

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**Disclosure.** The authors declare no conflict of interest.

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**Table 1. Clinical and laboratory characteristics of no-DN and DN in type 2 diabetic Patients**

	no-DN	DN	P*
Numbers	228	532	-
Age (years)	65.0±9.6	64.1±12.8	0.328
Age at diagnosis of diabetes (years)	49.7±10.4	54.3±13.0	<0.001
Sex (Male/Female)	101/127	281/251	0.033
Diabetes duration (years)	13.0 (11.0~17.8)	10.0 (4.0~15.0)	<0.001
Hypertension (%)	129 (56.6)	391 (73.5)	<0.001
Systolic blood pressure (mmHg)	138.1±18.9	143.2±20.2	0.001
Diastolic blood pressure (mmHg)	80.5±10.3	82.7±10.5	0.008
Fasting plasma glucose (mmol/L)	9.1±3.9	9.0±3.5	0.802
HbA1c (%)	9.2±4.2	8.9±2.7	0.437
Fasting plasma insulin (mU/l)	14.7 (9.2~21.0)	14.8 (9.8~22.8)	0.381
HOMA - $\beta$	63.8 (34.5~118.4)	62.6 (34.1~137.6)	0.653
HOMA - IR	5.3 (3.3~8.2)	5.7 (3.3~8.6)	0.446
Triglyceride (mmol/L)	1.3 (0.9~1.9)	1.6 (1.1~2.5)	<0.001
Total cholesterol (mmol/L)	4.8±1.2	4.9±1.4	0.125
Retinopathy (%)	98 (43.0)	284 (53.4)	0.008
Serum creatinine (umol/l)	64.0 (55.0~78.0)	84.0 (64.0~129.0)	<0.001
Smoking (%)	19 (8.3)	88 (16.5)	0.003
Hypoglycemic treatments			
Insulin (%)	111 (48.7)	275 (51.7)	0.467
OHA (%)	63 (27.6)	152 (28.6)	
Insulin + OHA (%)	54 (23.7)	105 (19.7)	

Data are presented as means  $\pm$  SD, medians (interquartile range) , or as n (%). Values of HOMA- $\beta$ , HOMA- IR and Fasting plasma insulin were calculated for no-DN (n=63) and DN (n=152) patients, who were not receiving insulin therapy. *P*\* values were obtained by unpaired Student's *t* test or chi-square analysis, as appropriate. no-DN (no diabetic nephropathy) ; DN (diabetic nephropathy) ; OHA (oral hypoglycemic agents); Insulin + OHA (Insulin in combination with OHA).

**Table 2. Clinical and laboratory characteristics of type 2 diabetic patients classified according to their PPARG-Pro12Ala genotypes**

	Pro/Pro	Pro/Ala	P*
Numbers	698	62	—
Age (years)	64.4±11.6	64.0±12.9	0.842
Age at diagnosis of diabetes (years)	53.1±12.2	51.3±14.3	0.286
Sex (Male/Female)	355/343	27/35	0.275
Diabetes duration (years)	10.0 (6.0~15.0)	11.0 (8.8~17.0)	0.163
Hypertension (%)	479 (68.6)	41 (66.1)	0.685
Systolic blood pressure (mmHg)	141.9±20.2	139.5±16.0	0.286
Diastolic blood pressure (mmHg)	82.1±10.5	81.5±10.0	0.696
Fasting plasma glucose (mmol/L)	9.0±3.6	9.5±3.8	0.473
HbA1c (%)	9.0±3.3	8.9±2.3	0.763
Fasting plasma insulin (mU/l)	14.6 (9.2~22.0)	16.8 (12.4~23.5)	0.140
HOMA- $\beta$	62.5(33.8~128.0)	72.1(50.3~189.9)	0.242
HOMA- IR	5.4 (3.2~8.6)	5.9 (3.8~10.4)	0.211
Triglyceride (mmol/L)	1.2 (0.9~1.6)	1.1 (0.8~1.6)	0.303
Total cholesterol (mmol/L)	4.9±1.3	5.1±1.4	0.129
Retinopathy (%)	354 (50.7)	28 (45.2)	0.402
AER (mg/24h)	80.8 (17.8~377.9)	21.4 (5.8~159.3)	0.001
Serum creatinine (umol/l)	76.0 (60.0~105.8)	68.0 (57.0~104.0)	0.515
Smoking (%)	97 (13.9)	10 (16.1)	0.628
Hypoglycemic treatments			
Insulin (%)	351 (50.3)	35 (56.5)	0.414
OHA (%)	197 (28.2)	18 (29.0)	
Insulin + OHA (%)	150 (21.5)	9 (14.5)	

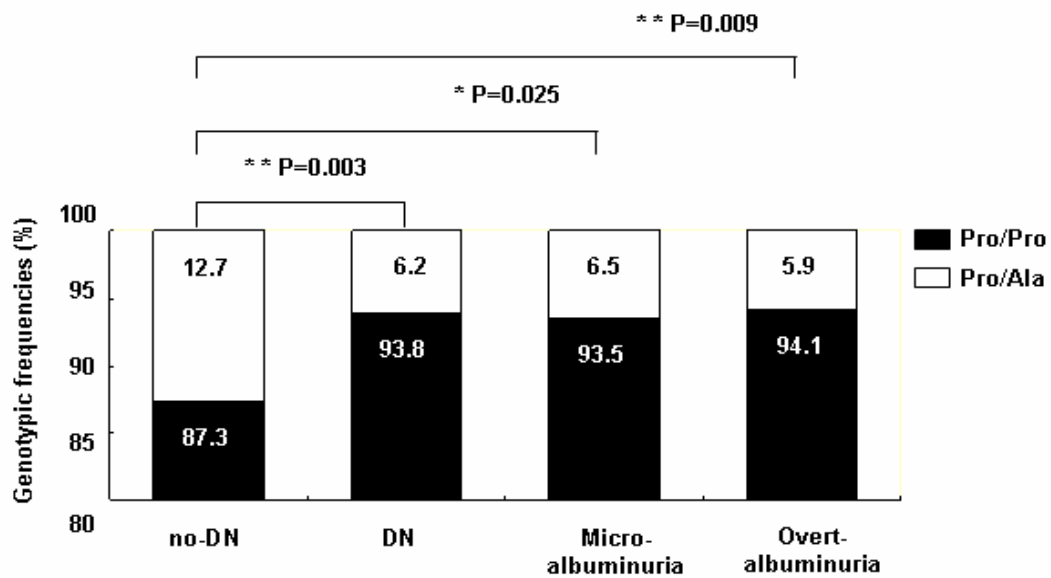
Data are presented as means  $\pm$  SD, medians (interquartile range) or as n (%). Values of HOMA- $\beta$ , HOMA -IR and Fasting plasma insulin were calculated for patients with Pro/Pro (n=197) and patients with Pro/Ala (n=18) respectively, who were not receiving insulin therapy. P\* values were obtained by unpaired Student's t test or chi-square analysis, as appropriate. OHA (oral hypoglycemic agents); Insulin + OHA (Insulin in combination with OHA).

**FIGURE LEGENDS**

**Figure 1.** Genotypic frequencies of PPARG-Pro12Ala polymorphism in type 2 diabetic patients with and without diabetic nephropathy. no-DN (no-diabetic nephropathy); DN (diabetic nephropathy).

**Figure 2.** Odds ratios of DN versus no-DN in different combinations of PPARG-Pro12Ala genotype and smoking status in a multivariate logistic regression analysis. The Odds ratios (OR) were adjusted for sex, age at diagnosis of diabetes, diabetes duration, hypertension, triglyceride, total cholesterol and HbA1c levels. A: Pro/Pro genotype; B: smoker. A-B-, nonsmokers without Pro/Pro genotype were considered as the reference (Ref.) group for determining the P values and ORs (95% CI). A+B- vs. A-B-, 2.06 (1.01-4.20); A-B+ vs. A-B-, 1.06 (0.20-5.52); A+B+ vs. A-B-, 4.52 (1.78-11.48). no-DN (no-diabetic nephropathy); DN (diabetic nephropathy).

**Figure 1.**



**Figure 2.**

