CLINICAL STUDY

Lack of association between peroxisome proliferator-activated receptor- γ -2 gene variants and the occurrence of coronary heart disease in patients with diabetes mellitus

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Abstract

Objective: Recent evidence indicates that peroxisome proliferator-activated receptor- γ (PPAR γ) is expressed at high levels in foam cells of atherosclerotic lesions, that PPAR γ agonists may directly modulate vessel wall function and that mutations in the PPAR γ -2 gene are associated with a reduced risk of coronary artery disease.

Methods: We investigated whether known variants in the PPAR γ -2 gene are associated with the occurrence of coronary heart disease (CHD) in 365 patients with type 2 diabetes, prospectively characterised for the presence or absence of CHD. The Pro115Gln, Pro12Ala, Pro467Leu, Val290Met mutations and two polymorphisms C478T and C161T of the PPAR γ -2 gene were examined using PCR, denaturing gradient gel electrophoresis and direct sequencing.

Results: The distribution of the Pro12Ala, Ala12Ala, C161T and T161T variants was not significantly different between patients with and without CHD, independent of the gender. The Pro12Ala ($P = 0.011$) and the Ala12Ala ($P = 0.006$) variant were associated with a higher body mass index (BMI) compared with the Pro12Pro genotype. A multiple logistic regression analysis introducing the typical risk factors for CHD (age, sex, hypertension, smoking, BMI >26 kg/m², elevated low density lipoprotein cholesterol and haemoglobin A_{1c} $>7\%$) identified age >60 , male gender, hypertension and a higher BMI, but not the PPAR γ -2 variants, as significant risk factors for CHD in our study groups.

Conclusion: The PPAR γ -2 genotype was not associated with an increased or reduced risk of the occurrence of CHD and can therefore not be regarded as an independent risk factor for CHD in patients with diabetes mellitus.

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Introduction

Peroxisome proliferator-activated receptors (PPARs) are nuclear receptors that function as transcriptional regulators of genes controlling lipid and glucose metabolism (1, 2). Three PPAR isotypes have been identified: α , β and γ . PPAR γ is mainly expressed in white adipose tissue (3) with lower levels expressed in many cell types, such as monocytes, differentiated human macrophages (2, 4, 5), skeletal muscle, vascular endothelial cells and epithelium of breast, colon and prostate (6). Furthermore, it could be shown that PPAR γ is involved in the gene regulation of inflammatory response (4), macrophage differentiation (5) and apoptosis induction (7). PPAR γ may promote atherosclerosis by stimulating the uptake of oxidised low density lipoprotein (LDL), a critical event in foam cell formation (5) and in addition PPAR γ may reduce expression of metalloproteinases,

such as MMP-9, which are implicated in plaque destabilisation (8–10). Moreover, atherogenic oxidised LDL particles could induce their own uptake through activation of PPAR γ (11) and expression of CD36, leading to foam cell formation (5). Moreover, there is evidence from studies using PPAR γ -deficient stem cells that PPAR γ is not essential for phagocytosis and inflammatory cytokine production in mature macrophages (12). However, recent reports suggest that PPAR γ -dependent and -independent counter-regulatory mechanisms exist, which offset the potential increase in oxidised LDL uptake mediated by PPAR γ in macrophages (4, 13–17). The potentially anti-atherogenic effects mediated by PPAR γ include cholesterol removal through ATP binding cassette transporter A₁ (ABCA1) (13) and anti-inflammatory effects such as the repression of phorbol ester-stimulated expression of interleukin-6 and -1 β , tumour necrosis factor- α , gelatinase and scavenger

receptor- α (4, 15). Furthermore, there is direct experimental evidence that PPAR γ activation can improve atherosclerosis (16) and human data showing that thiazolidinedione treatment led to a significant reduction of carotid artery wall thickness in patients with type 2 diabetes (18). These data suggest that PPAR γ is a candidate gene for a possible link between altered lipid and glucose metabolism, such as in patients with diabetes mellitus, and atherosclerosis development. Moreover, it has been recently shown that a C161T polymorphism in the PPAR γ -2 gene is associated with a reduced risk of coronary artery disease in patients who were consecutively referred for coronary angiography (19). Since it is difficult to assess the potential role of PPAR γ in atherosclerosis development in clinical studies, we chose the indirect approach, asking whether different PPAR γ -2 genotypes determine a different individual risk for the development of atherosclerosis.

We therefore investigated the association of known variants (19–22) in the PPAR γ -2 gene with the occurrence of coronary heart disease (CHD) in well-characterised patients with diabetes mellitus with and without CHD (CHD+ and CHD–).

Subjects and methods

Subjects

Three hundred and sixty-five consecutive patients with diabetes mellitus type 2 of the Third Medical Department of Leipzig University and the Leipzig Heart Centre were recruited for the study. All patients gave their informed consent for participation in the study. The patients were divided into two groups. Group 1 (CHD+, $n = 201$) and Group 2 (CHD–, $n = 164$). Type 2 diabetes was diagnosed according to American Diabetes Association criteria (23) by at least three times repeatedly >6 mmol/l elevated fasting or >11.1 mmol/l elevated 2 h oral glucose tolerance test plasma glucose concentrations. No patient had clinical evidence of type 1 diabetes and detectable glutamic acid decarboxylase and/or islet cell antibody. The diagnosis of CHD was established by a history of myocardial

infarction, coronary bypass operation or a significant stenosis ($>50\%$) of at least one coronary artery as defined by angiography. CHD– patients had neither a significant stenosis ($>50\%$) of at least one coronary artery as defined by angiography nor a history of acute myocardial infarction, ECG abnormalities and bypass surgery/coronary angioplasty. Smoking was defined as at least one cigarette, cigar or pipe per day. One pack year is defined as 20 cigarettes per day for 1 year. Hypertension was established if the blood pressure (in sitting position after 5 min of rest) was $>140/90$ mmHg or if patients were treated with anti-hypertensive drugs. Moreover, a comprehensive history of the patient's present medical treatment including angiotensin-converting enzyme inhibitors, beta-blockers, nitrates or lipid-lowering drugs was taken. Body mass index (BMI) was calculated as weight divided by squared height. Blood samples were taken after an overnight fast and after a 30 min supine position to determine glucose, HbA_{1c}, serum lipids and other standard laboratory parameters. Further clinical characterisation of the patients is shown in Table 1.

Preparation of genomic DNA and screening for PPAR γ -2 mutations

Genomic DNA was isolated from human leukocyte nuclei isolated from whole blood by proteinase K digestion using QIAamp DNA Blood Mini Kit (Qiagen Inc., Valencia, CA, USA). PCR amplifications of the segments with the mutations were all done in a total volume of 50 μ l containing PCR buffer (10 $\times$ Optiperm Buffer III; 500 mmol/l KOH pH 9.2 at 25 $^{\circ}$ C; 160 mmol/l (NH₄)₂SO₄; 0.1% Tween 20; 2 mmol/l MgCl₂), 5 μ l cDNA with 1 U CombiPool DNA Polymerase Mix *in vitro*, 0.5 mmol/l dNTPs and 1 μ mol/l sense and anti-sense primers. The PCR conditions for the Pro12Ala mutation, the Pro467Leu mutation and the silent polymorphisms CAC478CAT and C161T were: denaturation at 94 $^{\circ}$ C for 3 min, followed by 34 cycles of denaturation at 94 $^{\circ}$ C for 30 s, annealing at 56 $^{\circ}$ C for 30 s, extension at 72 $^{\circ}$ C and a final extension at 72 $^{\circ}$ C

Table 1 Clinical characterization of the study groups.

	Group 1 (CHD+)	Group 2 (CHD–)	P
Patients (n)	201	164	—
Age (mean (range))	67.1 (43–91)	63.3 (33–87)	<0.05
Female/male (n)	66/135	92/72	
Duration of diabetes (mean years (range))	8.93 (0.5–31)	9.13 (0.5–46)	0.2
Mean HbA _{1c} (% (range))	7.57 (4.8–14.2)	7.75 (4.6–14.1)	0.1
BMI (kg/m ² , (range))	28.68 (17.9–44.1)	28.77 (17.6–44.4)	0.49
Mean duration of CHD (years (range))	5.13 (0.5–23)	—	—
Mean age of CHD manifestation (years (range))	62.05 (33–86)	—	—
Hypertension (%)	76.6	62.2	<0.05
Smokers (% (range of pack years))	35.8 (1–150)	33.7 (1–80)	0.16
Mean LDL (mmol/l (range))	3.42 (0.98–7.78)	3.45 (0.78–7.01)	0.7

for 7 min. For the Pro115Gln and the Val290Met mutation, PCR was carried out as described above except for the number of cycles (36 cycles) and the annealing temperature (54 °C). The selection of all primers was based on the human genomic PPAR γ -2 sequence. All primer pairs span introns. The following primer pairs were selected: Pro12Ala: forward primer: 5'-GGC TCC TAA TAG GAC AGT GCC-3'; reverse primer: 5'-TAC CCT TAC ATA AAT GCC CCC-3', Pro115Gln: forward primer: 5'-TTG CCC TGT TGC CTT TTT AG-3', reverse primer: 5'-CAC CTA AAA AAG GGG TTC TGC-3'; Val290Met: forward primer: 5'-AGT CAT CCA CGT TTT CCC TG-3', reverse primer: 5'-TCC AAA ATT CTT TTG GCC AC-3', Pro467Leu and CAC478CAT: forward primer: 5'-TTT TTT GAC TGA ACC CCC TG-3', reverse primer: 5'-TGG AAG AAG GGA AAT GTT GG-3', C161T: forward primer: 5'-CAA GAC AAC CTG CTA CAA GC-3', reverse primer: 5'-TCC TTG TAG ATC TCC TGC AG-3'. As a screening method for the detection of the PPAR γ -2 gene mutations, denaturing gradient gel electrophoresis (DGGE) was used, which was shown to be more sensitive for the detection of somatic point mutations than direct sequencing (24). For DGGE, one GC clamped primer of each described primer pair was used. For mutation detection by DGGE, heteroduplexes between wildtype and mutant allele were generated. The optimal DGGE conditions were established for each DNA as previously described (24). The determined optimal DGGE conditions were as follows: Pro12Ala, Pro115Gln, Pro467Leu: 6% polyacrylamide gel containing a linear gradient of 20–40%, 3 h running time, Val290Met: 6% polyacrylamide gel containing a linear gradient of 20–60%, 6 h running time. Confirmation of the detected Pro12Ala and Ala12Ala variants and the detection of the silent polymorphisms CAC478CAT and C161T was performed using direct sequencing (ABI Prism Sequencer System; Applied Biosystems, Foster City, CA, USA).

Statistical analyses

The statistical analysis was performed with SPSS release 8.0.0 for Windows (SPSS Inc. 1989–1997). The χ^2 test was used to test frequencies of the Pro12Ala alleles in Groups 1 and 2. In addition, Student's *t*-test was used to test for differences between two or three different variables in the case of parametric distribution respectively. A Mann–Whitney U test was used in the case of non-parametric distribution. To obtain the odds ratios for specific risk factors for CHD, multivariate logistic regression analysis was performed.

Results

The frequency of the Pro12Ala variant was not different between CHD+ diabetic patients (Group 1: 11.5%,

Table 2 Distribution of the PPAR γ -2 gene genotypes in position 12 in Groups 1 and 2 (χ^2 test, $P = 0.36$).

	PPAR γ -2 genotype		
	Pro12Pro	Pro12Ala	Ala12Ala
Group 1, CHD+ (<i>n</i> (%))	174 (86.5%)	23 (11.5%)	4 (2.0%)
Group 2, CHD– (<i>n</i> (%))	140 (85.4%)	22 (13.4%)	2 (1.2%)
Total (<i>n</i> (%))	314 (86.1%)	45 (12.3%)	6 (1.6%)

males: 10.4%, females: 12.1%) and CHD– diabetic patients (Group 2: 13.4%, males 13.8%, females: 13.0%). The total allelic frequency of the Pro12Ala variant in both groups was 12.3% without differences between the genders (males: 12.1%, females: 12.7%). The frequency of the Ala12Ala variant was 2.0% in Group 1 and 1.2% in Group 2 (total frequency: 1.6%). All carriers of the Ala12Ala variant were male. The distribution of the different genotypes in the groups is shown in Table 2. The genotype distribution was not significantly different between the CHD+ and CHD– groups (χ^2 test, $P = 0.36$). Independently from the presence or absence of CHD, both the Pro12Ala ($P = 0.011$) and the Ala12Ala ($P = 0.006$) variant were associated with a higher BMI compared with the Pro12Pro genotype.

The frequencies of the C161T genotypes were not statistically significantly different between the group of CHD+ diabetic patients (Group 1: C161C: 85.6%, C161T: 12.9%, T161T: 1.5%) and the CHD– diabetic patients (Group 2: 82.9%, C161T 14.0%, T161T: 3.1%) (χ^2 test, $P = 0.28$). The frequency of the different genotypes was indistinguishable between males and females in both groups. There was also no association of the C161T polymorphism and BMI in either men or women.

The Pro467Leu, Val290Met, Pro115Gln mutations and the silent polymorphism CAC478CAT were not detected in any patient.

The HbA1c, LDL-cholesterol plasma concentrations, BMI, smoking habits or duration of diabetes were not significantly different between the groups (Table 1). In Group 1, patients with or without a history of acute myocardial infarction did not show statistically significant differences in the frequency of the Pro12Ala ($P = 0.8$), the Ala12Ala ($P = 0.6$), the C161T ($P = 0.2$) and the T161T ($P = 0.6$) variants. In addition, a multiple regression analysis was performed, introducing the typical cardiovascular risk factors age, sex, LDL-cholesterol plasma concentrations, hypertension, smoking and HbA1c as a marker for diabetes control and in addition the Pro12Ala, Ala12Ala, C161T and T161T genotypes of the PPAR γ -2 gene. This analysis showed that age >60, male gender, hypertension and a BMI >26 kg/m² are significant risk factors for CHD. However, there was no evidence that the PPAR γ -2 gene variant in position 12 as well as the polymorphism in

Table 3 Logistic regression analysis for cardiovascular risk factors associated with CHD in multivariate analysis (level of significance: $P < 0.05$). Total number of patients 365.

Cardiovascular risk factors	Odds ratio	P value	95% confidence interval
LDL (>4.1 mmol/l)	1.05	0.95	0.48–1.83
Age (>60 years)	3.47	<0.001	1.98–6.06
Gender (male)	3.20	0.001	1.63–3.87
Hypertension	3.62	<0.001	1.76–4.81
HbA _{1c} (7.0%)	0.81	0.44	0.49–1.37
Smoking	0.86	0.61	0.47–1.56
Pro12Pro	0.79	0.93	0.45–1.35
Pro12Ala	0.55	0.7	0.32–1.13
Ala12Ala	0.70	0.9	0.39–1.28
C161T	0.91	0.13	0.38–1.63
T161T	0.65	0.3	0.25–1.3
BMI (>26 kg/m ²)	3.10	0.0003	1.68–5.75

position 161 can be regarded as an independent risk factor for CHD (Table 3). Because the mean age was significantly higher in CHD+ diabetic patients (Group 1) as compared with CHD– diabetic patients (Group 2) ($P < 0.05$), patients were age-matched into subgroups of 5 year age range to control for the effect of age on a potential relationship between the PPAR γ genotype and CHD risk. In none of these subgroups could the PPAR γ genotype be identified as an independent CHD risk factor in the multiple regression analysis (Table 4). Moreover, to exclude the influence of the duration of diabetes as a confounding factor for the individual CHD risk, diabetes duration-matched subgroups were analysed. In 67 individuals (Group 1: $n = 35$, Group 2: $n = 32$) with a diabetes duration of ~ 5 years, we confirmed the absence of a relationship between the PPAR γ genotype and the CHD risk.

Discussion

Patients with diabetes mellitus have an increased risk of premature atherosclerosis (25, 26) and a 4-fold increased risk of dying from silent myocardial ischaemia/sudden heart death (27). Prediction of the individual

risk of developing premature atherosclerosis is important to select patients at high risk for more aggressive intervention strategies at an early stage of diabetes. The identification of genetic markers for an increased risk of atherosclerosis is one possible approach to determine an individual risk profile. PPAR γ is present in all major cells of the vasculature: human endothelial cells (28), vascular smooth muscle cells (29), macrophages (2, 4, 5) and resident atherosclerotic lesion macrophages (30). Furthermore PPARs are implicated in differentiation, foam cell formation (5), apoptosis (7) and inflammation control (4, 15) of macrophages. PPAR γ also plays an important role in the gene regulation of glucose and lipid metabolism. Therefore PPAR γ is a potential candidate gene for the link between diabetes mellitus and the premature atherosclerosis in patients with diabetes mellitus. This hypothesis is supported by the findings of Wang *et al.* (19), who reported an association of a C161T polymorphism in the PPAR γ gene with a reduced risk of coronary artery disease in patients with and without diabetes. Moreover, it was recently shown that treatment with troglitazone reduces the neointimal tissue proliferation after coronary stent implantation in patients with type 2 diabetes suggesting a causal involvement of PPAR γ in the development of atherosclerotic plaque (31). Therefore we investigated the possible relationship between the known variants in the PPAR γ -gene (Pro12Ala, Pro115Gln, Pro467Leu, Val290Met, C478T and C161T) and the occurrence of CHD in patients with diabetes mellitus in order to examine a possible genetic marker for the increased risk of patients with diabetes to develop CHD.

We did not find an association between the PPAR γ -2 genotype and CHD. We could not identify any patient with the rare Pro115Gln mutation. The absence of this variant was also reported in Danish (32), German (33) and American populations from several genetic backgrounds (34). Since there were also no carriers of the Pro467Leu and Val290Met mutations and the silent polymorphism CAC478CAT (35) in our study group, no conclusions regarding the potential

Table 4 Logistic regression analysis for the association between PPAR γ -2 variants and CHD risk in age-matched subgroups (level of significance: $P < 0.05$).

Age range group (years)	n (Group1/Group2)	Odds ratio (P-value)	
		Pro12Ala	C161T
<40	5 (0/5)	—	—
41–50	32 (6/26)	0.38 (0.52)	0.27 (0.79)
51–55	26 (14/12)	0.42 (0.7)	0.56 (0.8)
56–60	37 (21/16)	0.59 (0.64)	1.11 (0.09)
61–65	83 (46/37)	0.59 (0.6)	0.95 (0.5)
66–70	61 (38/23)	0.34 (0.1)	0.42 (0.28)
71–75	61 (45/16)	0.29 (0.44)	0.96 (0.64)
76–80	44 (24/20)	0.62 (0.21)	0.66 (0.6)
>80	16 (7/9)	0.36 (0.18)	0.75 (0.2)

importance of these mutations for an altered CHD risk can be drawn. The Pro467Leu and the Val290Met mutation could at least indirectly increase the CHD risk, because a causal link between these variants and early onset hypertension has been suggested (20).

Wang *et al.* (19) reported that the C161T polymorphism in the PPAR γ -2 gene is associated with a decreased individual CHD risk. We could not confirm this finding in our study population. One explanation for these divergent findings could be that our study was exclusively performed with patients with diabetes, whereas Wang *et al.* (19) mainly studied patients without diabetes. Moreover, the frequency of the C161T polymorphism was lower in our study (Group 1: 12.8%, Group 2: 14.1%) as compared with the C161T frequency (27.7%) reported in the previous study (19). This might suggest that the C161T polymorphism is less frequent in patients with diabetes. Further studies are necessary to compare the frequency of this polymorphism in subjects with and without type 2 diabetes and to assess a possible relationship between the C161T polymorphism and the risk of developing type 2 diabetes.

The frequency of the Pro12Ala variant in the PPAR γ -2 gene was similar in our study group to those reported in previous studies investigating obese patients with type 2 diabetes (32, 33, 35–37). However, our logistic multiple regression analysis only identified an age >60, male gender, hypertension and a higher BMI, but not the Pro12Ala or the C161T variants of the PPAR γ -2 gene, as independent risk factors for CHD. Since only patients with type 2 diabetes were selected and since there were no differences in HbA_{1c} values, LDL-cholesterol concentration and the frequency of smoking between the CHD+ and CHD– groups, the cardiovascular risk factors elevated HbA_{1c} values, elevated LDL-cholesterol concentration and smoking were not identified as independent risk factors in our study population. Therefore, we can exclude these cardiovascular risk factors as confounding factors in the analysis of the relationship between PPAR γ genotype and CHD risk.

Since the number of homozygous Ala12Ala and T161T patients was small, a statistically significant association of this genotype with an altered risk for the occurrence of CHD could not be established. Considering the multiple factors contributing to variable association study results as described recently (38) the statistical power (>80%) of our study was only adequate to detect an association between PPAR γ -2 gene variants and the CHD risk for the frequent Pro12Ala (frequency >12.5%) variant and the C161T (frequency >13.4%) polymorphism. More than 8000 patients would be needed for the detection of a possible association between the Ala12Ala variant and the CHD risk according to calculation based on allele frequency, effect size and population variance.

Moreover, the Pro12Ala polymorphism has been variably associated with either increased (36),

decreased (22) or unchanged (32, 33) BMI. In our study we found an association of the Pro12Ala variant with an increased BMI. In contrast to Japanese men (39), but in accordance with results from previous studies among Caucasians (36, 37), the homozygous Ala12Ala variant was also associated with an increased BMI. However, the effect of the Ala12Ala variant seems to be associated with the degree of obesity (37). Among normal weight or slightly overweight subjects, the Ala12Ala variant was associated with lower than average BMI (35), whereas the association between the Ala12Ala variant and an increased BMI was more pronounced in very obese patients (36, 37). There is no obvious explanation for the discrepancy between lean and obese subjects but the results could indicate a variable interaction of the Ala allele with other genetic and environmental factors influencing BMI (32).

We investigated a heterogeneous, high-risk hospitalised patient population. Despite the sufficient statistical power for the frequent Pro12Ala and C161T variants (>80%), our study results should be interpreted in the context of the studied individuals. CHD+ patients (Group 1) were significantly older than the CHD– patients (Group 2) and subsequently in our analysis the age is one of the main risk factors for the development of CHD. The age difference is mainly due to a lower number of younger patients (<50 years) in Group 1. Therefore, we introduced age-matched subgroups (patients were matched into groups of 5 year age difference, e.g. from 51–55 years up to older than 80 years) (Table 4) to control for the effect of the age on the potential relationship between PPAR γ genotype and CHD risk. However, in none of the age-matched subgroups could the PPAR γ genotype be identified as an independent CHD risk factor. Moreover, we excluded in our study population an effect of the diabetes duration on the impact of the possible association between PPAR γ polymorphisms and CHD risk in a diabetes duration-matched subgroup analysis.

In conclusion, the Pro12Ala and Ala12Ala variants and the C161T polymorphism in the PPAR γ -2 gene are not associated with an increased or reduced risk of the occurrence of CHD in our study and can therefore not be regarded as an independent risk factor for CHD. Since both variants are associated with an increased BMI and since our logistic multiple regression analysis identified, apart from an age >60, male gender and hypertension, a BMI >26 kg/m² as a CHD risk factor, the Pro12Ala and Ala12Ala variants could therefore indirectly contribute to a higher CHD risk in these patients.

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