*J Nutr Health Aging. 2019;23(2):175-180*



# ASSOCIATION BETWEEN PPARS GENE FUNCTIONAL POLYMORPHISMS AND ISCHEMIC STROKE IN CHINESE UYGHUR POPULATION

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**Abstract:** PPARγ and PPARα belong to a receptor family of ligand-activated transcription factors involved in the regulation of inflammation, cellular glucose uptake, protection against atherosclerosis and endothelial cell function. Through these effects, they might be involved with the ischemic stroke (IS). We recruited 100 IS patients diagnosed by CTs or/and magnetic resonance imaging (MRI) and 100 normal healthy controls from Chinese Uyghur Population to assess the nature of the functional polymorphisms of PPARs and any links with IS in this unique population which has 60% European ancestry and 40% East Asian ancestry. We found that the Ala allele of the PPARγ Pro12Ala polymorphism was more common in controls than IS subjects (P = 0.008, corrected for multiple testing) in the Uyghur Population. Pro/Ala carriage may be associated with a decreased risk of IS in Uyghurs (OR 0.542, 95% CI 0.346-0.850). Additionally, the 162Val allele frequency at the DNA-binding region of PPARα was extremely rare in Chinese Uguhur IS patients and controls. Our population and ethnic-based study demonstrates that the 162Val allele frequency was extremely low in the Chinese Uyghur Population different from Some European and African populations and the PPARγ 12 Pro/Ala resulting in an amino acid exchange in N-terminal sequence may be an independent protective factor for IS in the Chinese Uyghur Population.

**Key words:** Ischemic stroke, functional polymorphisms, peroxisome proliferator-activated receptors (PPAR)-s, Uyghur Population.

## Introduction

Ischemic stroke (IS) is a major cause of morbidity and mortality in many developed and developing countries. Chronic low-grade inflammation and activation of the innate immune system are closely involved in the pathogenesis of IS (1, 2). Peroxisome proliferator-activated receptor γ&α (PPARγ&PPARα) play key roles in the regulation of the inflammation, adipocyte differentiation and lipid biosynthesis (3). PPARγ regulates the expression of many inflammatory cytokines and adipose-specific genes via binding of a heterodimer of PPARγ and R×R (Retinoid × receptor) to regulatory response elements in target gene promoters (4). PPARα, a nuclear receptor which regular the expression of genes, is involved in peroxisomal and mitochondrial b-oxidation pathways such as fatty acid uptake and the catabolism of circulating triglyceride (5, 6). Through these effects, PPARγ and PPARα might be play crucial roles in the development and progression of IS.

The Leu162Val polymorphism in PPARα, a functional variant which was identified in the DNA-binding domain of human PPARα (10), has been suggested to possess an increased ligand-dependent transcriptionalactivity and serum concentrations of triglyceride (7-11). The common Leu162Val polymorphism in the gene encoding PPARα has inconsistently shown association with quantitative traits related to obesity, type 2 diabetes, and coronary heart disease (7, 12, 13). However, no study demonstrates an effect of the Leu162Val polymorphism on IS.

The PPARγ Pro12Ala polymorphism results in a Pro to Ala amino acid exchange in the N-terminal sequence at codon 12 which is only present in the PPARγ isoform (4). PPARγ is primarily expressed in adipose tissue. In vitro, the PPARγ Pro12Ala variant is a less active transcription factor, resulting in lower transcription levels of target genes (14-16). Several polymorphisms in the PPARγ gene have already been described, of which the PPARγ Pro12Ala polymorphism seem to be relevant in a variety of diseases such as type 2 diabetes, atherosclerosis, and Alzheimer's disease. However, their role for IS has not yet been investigated (17-20).

In the current study, we screened 100 patients with clinically definite IS and 100 age- , sex-, ethnic-matched healthy controls from Chinese Uyghur subject panel for studying the association of PPARα Leu162Val and PPARγ Pro12Ala polymorphisms with IS.

## Methods

### *Study population*

Our study was approved by the Institutional Review Board of Shenzhen Center for Disease Controls and Prevention, and signed informed consents were obtained from all participants or from patients' representatives if direct consent could not

*Received May 23, 2018*
*Accepted for publication August 7, 2018*
Published online November 22, 2018, http://dx.doi.org/10.1007/s12603-018-1140-3

be obtained. The experiment methods were carried out in accordance with the approved guidelines and regulations, and all experimental protocols were approved by the Institutional Review Board of Shenzhen Center for Disease Controls and Prevention.

We enrolled inpatients attending the stroke units of five large general hospitals randomly from 2003 to 2010 (Fixed stroke research institutions) in Xinjiang. The diagnosis of IS was established using the World Health Organization, International Classification (21) and stroke subtypes were defined using the Oxfordshire classification (22, 23). Screened by head CTs or/and magnetic resonance imaging (MRI) with established reports, a total of 100 subjects presenting within 24 hours of symptom onset clinically diagnosed as IS were enrolled in the case-control study. Additionally, 100 age-, gender- and ethnicity-matched normal healthy controls were selected from healthy volunteers by stratified random cluster sampling from four local community-based populations. Subjects with a history of stroke, Alzheimer's disease, brain aneurysm, dementia, dystonia, Parkinson's disease or inflammatory disorders were excluded from the control group. A structured questionnaire was used to record general information, clinical history of IS and associated clinical parameters, and epidemiological data.

***Sample processing***

After obtaining informed consent from both groups, 5mL blood samples were taken in ethylenediamine tetraacetic acid (EDTA) and plain vials. Genomic DNA was extracted from the peripheral blood leucocyte pellet using a DNA extraction kit (AXYGEN, California, USA). DNA samples were stored at -80°C before use.

***Genotyping of the PPARα Leu162Val and PPARγ Pro12Ala genomic variants***

Genotyping of PPARα Leu162Val and PPARγ Pro12Ala were performed with Taqman SNP allelic discrimination by means of an ABI 7500 (Applied Biosystems, Foster City, CA), in 96-well format. PCR reactions were carried out in reaction volume of 5μl containing 5ng DNA, 2.5μl 2× Taqman universal PCR Master MixNo AmpErase UNG (Applied Biosystems), 0.125μl 40×Assay Mix. PCR conditions included 95°C for 10 min, followed by 40 cycles of 15 s at 92°C and 1 min at 60°C.Two blank controls (DNA hydration solution) and two replicate quality control samples were included in each 96-well format, and two replicate samples were genotyped with 100% concordance. The intensity of each SNP met the criteria of three clear clusters in two scales generated by SDS software (ABI).

To improve the genotyping quality and validate our results, a random selection of 10% of the samples were re-genotyped by laboratory personnel not otherwise involved in the study, and the results were found to be reproducible with no discrepancies noted.

***Biological variables determination***

Total Triglyceride (TG), total Cholesterol (TC), HDL and fasting blood glucose(FBG) were assayed using standard laboratory procedures in the department of clinical laboratory at the local Hospital.

***Statistical analysis***

Allele frequencies, genotype frequencies and carriage rates of the alleles in all the groups were compared by the Fisher exact test using SPSS software (version 12.0, SPSS Inc., Chicago, Illinois, USA). Data on quantitative characteristics were expressed as means ± SD. Comparisons between groups were made with the $\chi 2$ test (nominal data) or analysis of variance (ANOVA), with post hoc t-tests using pooled standard deviations (interval data). Allele frequency was calculated as the number of occurrences of the test allele in the population divided by the total number of alleles. The carriage rate was calculated as the number of individuals carrying at least one copy of the test allele divided by the total number of individuals. All P values were two sided, and differences were considered statistically significant if $P<0.05$. Hardy–Weinberg equilibrium was tested by the $\chi 2$ method. Odds ratios (OR) at the 95% confidence interval (CI) were determined to describe the strength of any associations between IS and gene polymorphism using multivariate logistic regression modeling.

## Results

A total of 100 IS patients and 100 healthy controls were evaluated for the PPARs polymorphisms study from Uyghur subject panel of China. The ratio of males to females was 58:42 in the Uyghur subjects. There were no significant differences in age between cases and controls in study populations (P=0.498). There was a significant difference in waist-to-hip ratio (WHR) between the patients and controls in the Uyghur Population (P=0.001). There was a significant difference too in body mass index (BMI) ($P<0.05$) between the patients and controls in the Uyghur Population. The results suggest that BMI and WHR increased the risk of IS in Uyghur ethnic group. As shown in Table 1, the Uyghur IS group had a higher prevalence of conventional risk factors for vascular diseases, including BMI, WHR, history of hypertension, heart diseases and negative events compared to the Uyghur control group. The results suggest that BMI, WHR, hypertension, heart diseases, negative events may increase the risk of IS. However, there was a trend for Cholesterol and HDL to be protective factors for IS [Table 1].

Table 2 shows the distributions of the genotypes and allelic frequencies of PPARα Leu162Val and PPARγ Pro12Ala polymorphisms. There were no statistically significant difference in the distribution of PPARα Leu162Val polymorphism between the patients and controls in Uyghur ethnic group ($P>0.05$). The Val allele frequencies at the 162 DNA-binding domain of PPARα were extremely low in the

Chinese Uyghur Population. However, there were significant differences in the distribution of Pro12Ala polymorphism between the patients and controls in the Uyghur group (P<0.01). This suggests that PPARγ Pro12Ala may be a protective factor for IS in Uyghur Chinese. With PPARγ Pro/Pro as the reference genotype, PPARγ Pro12Ala had an odds ratio for IS of 0.57 (P<0.01) in Uyghur people. The Uyghur patient group had an decreased frequency of the Ala allele compared to the Uyghur control group and the odds ratio for the Ala allele, as opposed to the Pro allele, was 0.63 in Uyghur Chinese (P<0.05). The PPARγ Pro/Ala polymorphism was Hardy–Weinberg equilibrium in Uyghur ethnic groups (P>0.05).

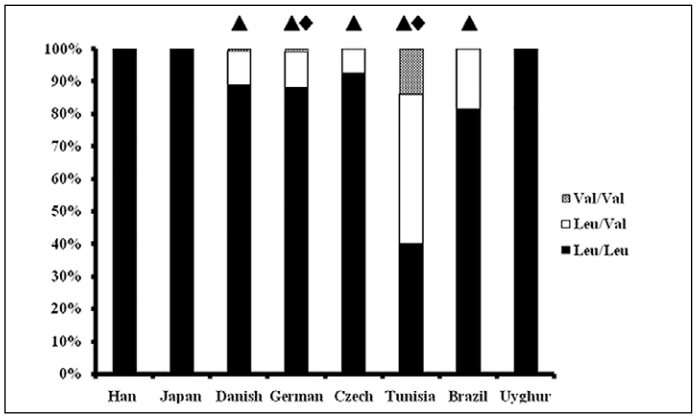
**Table 1**
Demographic characteristics and distribution of traditional risk factors in Chinese Uyghur population

| Characteristic | Uyghur | | P Value[c] |
|---|---|---|---|
| | Cases (n=100) | Controls (n=100) | |
| Gender (M/F) | 58/42 | 58/42 | |
| Age (years) | 64.12±9.36 | 63.18±10.19 | 0.498 |
| BMI (Kg/m$^2$) | 24.51±3.47 | 23.56±2.73 | 0.040 |
| WHR | 0.89±0.07 | 0.80±0.06 | 0.0001 |
| FH of hypertension | 36.00% | 46.00% | 0.151 |
| FH of diabetes | 15.00% | 25.00% | 0.077 |
| History of hypertension[a] | 59.00% | 11.00% | 1.111×10-12 |
| History of diabetes | 10.00% | 8.00% | 0.621 |
| History of heart diseases | 8.00% | 0.00% | 0.007 |
| Smoking[b] | 17 | 19 | 0.713 |
| Current alcohol drinker | 16.00% | 26.00% | 0.083 |
| Negative events | 14.00% | 1.00% | 0.0005 |
| Tea consumption | 98.00% | 100.00% | 0.155 |
| Triglyceride (mmol/l) | 1.69(1.07) | 1.66(0.92) | 0.849 |
| Cholesterol (mmol/l) | 4.53(1.24) | 5.65(1.23) | 0.0002 |
| HDL (mmol/l) | 1.06(0.37) | 1.39(0.38) | 0.00001 |
| LDL (mmol/l) | 2.70(0.77) | 2.78(0.53) | 0.767 |
| FBG (mmol/l) | 6.51(2.16) | 5.35(1.71) | 0.0004 |

M male, F female, BMI body mass index, WHR Waist hip ratio, FH family history; FBG fasting blood glucose.

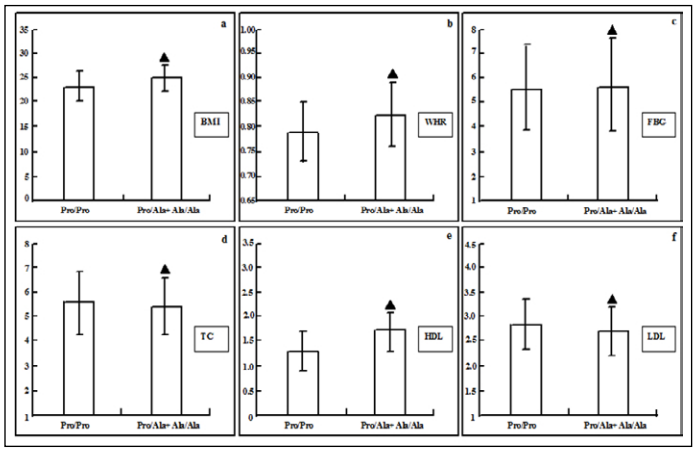
In order to analyze the genotype frequency distribution background between different population. We compared the PPARα Leu162Val genotype frequency distribution between different ethnic groups based on the published research data and present study in Figure 1 (24-28). The PPARα 162Leu/Val genotype was more frequent in Danish, German, Czech, Tunsia, Brazil population than in Asian Uyghurs, Hans and Japans (All P<0.05). The PPARγ 162 Val/Val genotype was more frequent only in German and Tunsia population than in Uyghurs, Hans and Japans (All P<0.05). The PPARα 162 Leu/Val genotype was absent in Uyghurs, Hans and Japans. Meanwhile, The PPARα 162 Val/Val genotype was absent in Uyghurs, Hans, Japans, Germans and Brazils.

**Figure 1**
PPARα 162 Leu/Val genotype frequency distribution between various populations




▲ Compared with Uyghurs and Japans on the Leu/Val genotype frequency; P<0.05; ♦ Compared with Uyghurs and Japans population on the Val /Val genotype frequency,P<0.05.

**Figure 2**
The association of BMI, WHR, FBG, TC, HDL and LDL of controls with Pro12Ala genotypes in the Uyghur (a,b,c,d,e,f) ethnic group




▲ indicate P>0.05(Pro/Pro VS Pro/Ala + Ala/Ala).

Furthermore, we also analyzed the association of BMI, WHR, FBG,TC,HDL and LDL of controls with Pro12Ala genotypes, however, we failed to find any significant association with any of these parameters in Uyghur Population (All P>0.05) [Fig 2].

Table 3 shows a multiple logistic regression model for evaluating the relative effects of PPARγ Pro12Ala polymorphism on the risk of IS. The relationship between Pro12Ala polymorphism and IS was further assessed by multivariate logistic regression analysis, and variables were

entered into a logistic regression model if they were statistically significant following univariate analysis. In the Uyghur group, after adjustment for Hypertension, Diabetes, Smoking, Tea drinking, Alcohol drinking, BMI and WHR, the PPARγ Ala allele was associated with an decreased risk of IS, with an overall OR of 0.542 and 0.555 when assuming Additive and Dominant models of inheritance, respectively (All P<0.01).

**Table 2**
PPARα Leu162Val and PPARγ Pro12Ala genotype and allele distributions between cases and controls in Chinese Uyghur Population

| | Uyghur | | | |
|---|---|---|---|---|
| | **Cases n=100** | **Controls n=100** | **P Value** | **OR (95%CI)** |
| PPARα (162) | | | | |
| Leu/ Leu | 100 | 100 | | |
| Leu/Val | 0 | 0 | --- | --- |
| Val /Val | 0 | 0 | --- | --- |
| PPARγ alleles | | | | |
| Leu | 200 | 200 | | |
| Va l | 0 | 0 | --- | --- |
| MAF | 0 | 0 | --- | --- |
| PPARγ(12) | | | | |
| Pro/Pro | 606 | 580 | | |
| Pro/Ala | 40 | 67 | 0.007 | 0.571(0.380-0.859) |
| Ala/Ala | 2 | 1 | 1.000 | 1.914(0.173-21.167) |
| Pro/Ala + Ala/Ala | 42 | 68 | 0.010 | 0.591(0.396-0.883) |
| PPARγ alleles | | | | |
| Pro | 1252 | 1227 | | |
| Ala | 44 | 69 | 0.016 | 0.625(0.425-0.920) |
| MAF | 3.40% | 5.32% | | |

MAF: Minor Allele Frequency

**Table 3**
Association of PPARγ Pro12Ala polymorphisms on the risk of IS in Chinese Uyghur Population

| | Uyghur | | | |
|---|---|---|---|---|
| | **Unadjusted OR(95%CI)** | **P value** | **Adjusted OR(95%CI)** | **P value** |
| Pro12Ala | | | | |
| Recessive | 2.003 (0.181-22.415) | 0.563 | 2.005(0.183-22.420) | 0.560 |
| Additive | 0.625 (0.425-0.920) | 0.016 | 0.542(0.346-0.850) | 0.008 |
| Dominant | 0.591 (0.396-0.883) | 0.010 | 0.555(0.356-0.864) | 0.009 |

Multivariate logistic regression analysis after adjustment for Hypertension, Diabetes, Smoking, Alcohol drinking, Tea drinking, BMI, WHR, TG and TC.

## Discussion

Earlier studies have reported that acute infections, accompanied by acute phase inflammation reactions and prothrombotic state, have been associated with an increased risk of acute vascular events such as myocardial infarction and IS (29-31). The susceptibility to infection/inflammation and/or risk of atherosclerosis and IS events have been shown to be associated with genetic variations in genes and cytokine receptor genes (32-34). However, data relating PPARα Leu162Val and PPARγ Pro12Ala polymorphism to IS are lacking or controversial (35-37).

Some previous studies have found a unique association between the Leu162Val polymorphism and increased fasting serum levels of triglyceride and total cholesterol [24]. Triglyceride and total cholesterol also contributes to ischemic events through the promotion of atherosclerosis [38]. We assume that this association may thereby influence the incidence and outcome of IS. In addition, it has been reported that PPARα Leu162Val polymorphisms are associated with other inflammation- and atherosclerosis-related diseases, such as type 2 diabetes, obesity, coronary artery disease and dyslipidaemia (7, 12, 13). Recently, the Leu162Val polymorphism has been linked with increased risk of Non-alcoholic fatty liver disease (39). However, we failed to demonstrate an association between functional polymorphism of PPARα Leu162Val with the occurrence of IS in Chinese Uyghur Population. Interestingly, we found that the Val allele frequency at the DNA-binding domain of human PPARα was extremely low in Uyghur IS patients and controls which were similar to Japans, Danish, Czech and Brazil populations but not similar to German and Tunisia populations. The reason for the rarity of Val allele in the Chinese population is unclear and needs further ethnicity-specific studies.

In the present study, the Pro/Ala genotype of the PPARγ Pro12Ala polymorphism was associated with IS significantly in the Uyghur Population. Previous studies have already characterized the PPARγ Pro12Ala polymorphism in a variety of metabolic disorders, such as hyperlipidemia, insulin resistance, type 2 diabetes mellitus, atherosclerosis and diabetic nephropathy (19, 20, 40, 41), most of these studies observed a protective effect in carriers of the Ala allele (20, 41-43). Especially, the effect of PPARγ Pro12Ala polymorphism on diabetes has been confirmed by researchers (44). However, we did not find any significant association of Pro12Ala with FBG but observe a significant change in the strength of association between PPARγ Pro12Ala and IS, after adjusting for diabetes, suggesting that the role of PPARγ Ala allele in diabetes does not affect the genetic associations we report here.

In addition, the PPARγ polymorphism might influence inflammatory processes, as the Ala allele was associated with significantly lowered leukocyte counts (42) and with decreased levels of markers of systemic inflammation in renal disease (45). In this context, it should be mentioned that most of

the atherosclerosis-associated disorders with the Pro12Ala polymorphism have a key inflammatory component (46).

To our knowledge, an association of the PPARγ Pro12Ala polymorphism with IS has not yet been shown. However, an impact of PPARγ on disease severity has already been demonstrated in the wild-type mice model of IS (47, 48), as PPARγ deficient heterozygous knockout mice exhibit an aggravated disease phenotype accompanied by increased T-cell expansion and pronounced immune responses, thus demonstrating a critical role of PPARγ for the regulation of CNS autoimmunity (49). Several studies suggest that the PPARγ Pro12Ala polymorphism might influence PPARγ activity, as it generates a CCA to GCA missense mutation in codon 12 of exon 1 of PPARγ, which encodes for the ligand-independent DNA-binding domain (16). The protective effect of this polymorphism could therefore be due to an alteration of receptor activity. Interestingly, transient transfection assays demonstrated that the Ala-variant exhibited altered binding activity to PPARγ-responsive DNA elements as compared to wild-type PPARγ (16, 50). Moreover, several PPARγ target genes have been found to be differentially expressed after over-expression of the Ala variant in comparison to over-expression of the wild-type protein; which again suggests that the Pro-to-Ala exchange might alter the transcriptional activity of PPARγ (16). Another intriguing hypothesis is protection against inflammation-induced loss of PPARγ conferred by the Ala/Ala genotype. It has been shown that PPARγ activation prevents synthesis and release of cytokines (interleukin-6 and tumour necrosis factor α) or induction of some inflammatory mediators such as COX-2 (cyclo-oxygenase 2) and adhesion proteins. These effects on inflammation explain why activation of PPARγ by synthetic ligands reduces inflammation in different tissues and in different animal models of inflammatory diseases such as vascular inflammation of atherosclerosis, inflammatory bowel disease and arthritis (51, 52). Therefore, the protective effect of the Ala/Ala genotype could also involve a protection from inflammation-induced loss of PPARγ, thus favoring the balance towards anti-inflammatory signaling and reducing the risk of IS.

Strengths of the current study are a relative scarce sample in the Uyghur Population, rigorous methods used to diagnose IS including formal assessment of the reliability of interpretations of head CTs or MRI findings, and a biologically plausible a priori rationale for choosing the candidate genes. Our study also has some limitations. First, it is a case-control study and a selection bias could not be completely excluded for the group of patients with IS. Second, although controls with diseases potentially related to ischemic disorders were debarred from entry to the study, there may have been enrollees with undiagnosed disease, so a selection bias cannot be categorically excluded. Third, it is possible that our findings might apply only to the Chinese population. Finally, due to the case-control study design, we were not able to analyze the association of PPARs gene functional polymorphisms with stroke severity was using NIHSS.

Taken together, we conclude that the 162Val allele frequency at the DNA-binding domain of human PPARα was extremely low in the Chinese Uyghur Population not similar to some European and African populations. The PPARα Leu162Val is unlikely to contribute significantly to a susceptibility to, or affect the progression of IS in Uyghur Population. Moreover our findings lend support to the notion of an independent protective effect of the Ala allele of the PPARγ Pro12Ala on the risk of IS in the Uyghur Population. Further explorations with larger, more ethnically diverse populations are warranted to better shed light on the functional properties of this receptor on the risk of IS, as well as the complicated pathophysiology mechanisms precisely. These findings may affect the diagnosis and treatment of IS.

*Acknowledgements:* This study was financially supported by research funds (No.81302497&81573237) from National Natural Science Foundation of China (NSFC) and Hubei Natural Science Foundation (No. 2013CFB056&2016CFB403&2017ADC061) and Hubei Province's outstanding medical academic leader program and Shenzhen Municipal Science and Technology Innovation Committee(No.KJYY20170413162318686) as well as Hubei young talent program (20171102). We are grateful to all participants of this study, and we must say "thank you" to Yijie Geng for their technical assistance and Frank Xu for his language help.

*Competing financial interests:* The authors declare that they have no competing interests.

*Ethical standard:* The study was carried out in accordance with the Declaration of Helsinki of the World Medical Association and received ethical approval from the local Institutional Review Board.

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