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Genetic variation of the intestinal fatty acid-binding protein 2 gene in carotid atherosclerosis

Pär Wanby^a, Petter Palmquist^a, Lars Brudin^b and Martin Carlsson^c

Abstract: The alanine (A) to threonine (T) substitution at codon 54 of the intestinal fatty acid-binding protein 2 (FABP2) has been associated with dyslipidaemia and other characteristics of the metabolic syndrome, which in turn is a risk factor for cerebrovascular disease. The aim of this study was to investigate whether the A54T polymorphism in the FABP2 gene is associated with internal carotid artery (ICA) stenosis in stroke patients. Swedish subjects initially diagnosed with acute cerebrovascular disease ($n = 196$) that had been assessed with ultrasound of the carotid arteries were identified and grouped depending on whether a stenosis was found. The subjects were genotyped for the A54T polymorphism using a PCR-RFLP method. In a multivariate logistic-regression analysis, where known risk factors for atherosclerosis were fixed (diabetes, systolic blood pressure, age and smoking), having the FABP2 T allele was a significant risk factor for ICA stenosis (odds ratio 2.9; 95% confidence interval, 1.1–7.7; $p = 0.04$) together with diabetes (odds ratio 4.9; 95% confidence interval, 1.8–14; $p < 0.01$). Age, smoking and blood pressure did not reach statistical significance. In conclusion, our result supports the hypothesis that the FABP2 A54T polymorphism is associated with ICA stenosis.

Key words: carotid atherosclerosis; FABP2; genetics; polymorphism

Introduction

Internal carotid artery (ICA) stenosis is a sign of advanced atherosclerotic disease, and ICA stenosis is recognized as a major risk factor of stroke. Conversely, carotid endarterectomy is effective in the prevention of stroke secondary to severe ICA stenosis.¹ Fatty acid-binding protein 2 (FABP2) is an abundant cytosolic protein expressed exclusively in the enterocytes of the proximal small intestine, and FABP2 is believed to be involved in fatty acid absorption and intracellular transport of dietary long-chain free fatty acids (FFAs).^{2,3} A common gene polymorphism at codon 54 results in replacement of alanine (A) with threonine (T).⁴

In vitro, the T-containing protein has a 2-fold greater affinity for long-chain FFAs.^{4,5} This altered affinity for FFAs in the intestine is believed to affect the absorption, and consequently the fatty acid

composition of serum lipids. In an earlier study using genotype-discordant sibling pairs we found that siblings with more T54 alleles had higher triglycerides and cholesterol concentrations than their siblings with more A54 alleles.⁶ In other studies the T54 allele has been associated with insulin resistance,^{4,7} obesity and serum lipid abnormalities, including higher fasting triglyceride concentrations⁸ and elevated triglyceride⁹ and FFA¹⁰ concentrations after an oral fat load. A few other case-control studies could not replicate these data.^{11,12} Stroke has been associated with the metabolic syndrome and, in a recent study,¹³ with hypertriglyceridemia in particular. In addition, coronary restenosis after balloon angioplasty has been associated with a different polymorphism in the FABP2 gene.¹⁴ In our earlier study we suggested that the T54 allele might increase susceptibility to stroke, as we found a higher parental prevalence of stroke in TT and TA genotype carriers when compared with AA genotype carriers.⁶ Several reports have pointed out an association between increased levels of postprandial triglyceride-rich lipoproteins and the presence of or development of coronary artery disease.^{15–17} Since other earlier studies have shown that the FABP2 T54 allele is associated with postprandial lipemia,^{7,9,10} and since postprandial lipemia is associated with carotid atherosclerosis,^{18–21} we raised the hypothesis

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that the FABP2 gene A54T polymorphism would be associated with an increased risk of ICA stenosis.

Simpler genetic effects probably underlie the development of ICA stenosis compared with stroke and different stroke subgroups. In this study we therefore chose to study the FABP2 gene polymorphism in relation to ICA stenosis instead of studying the effect on stroke itself.

Methods

Subjects

Between June 2000 and November 2002, 497 patients initially diagnosed with stroke or a transitory ischemic attack (TIA) were, upon their admission to the acute stroke unit, recruited to the Kalmar Stroke Study. Those who were investigated using ultrasound of the carotids were identified and included in the present study. Depending on the result of the ultrasound assessment, they were either classified as cases (patients with ICA stenosis, $n = 25$) or as controls (patients without ICA stenosis, $n = 171$). Of the study group ($n = 196$), 133 (68%) had received a final diagnosis of ischemic cerebral infarction, 30 (15%) had suffered a cardio-embolic infarction, 29 (15%) had had a TIA, and the remaining four patients (2%) had been found not to have suffered a stroke or TIA.

As a reference of FABP2 genotype frequency in a healthy population we used 158 genotyped blood donors from the same hospital (age = 55.4 ± 3.9 years, 83 males, 75 females) whose internal carotid arteries had not been assessed with ultrasound. The blood donors were free from drugs and without a history of coronary, peripheral or cerebrovascular disease. Blood samples other than those used for DNA genotyping were not obtained. All participants gave informed consent to participate in the study, which received the approval of the Ethical Committee of Linköping University, Sweden.

The County Hospital of Kalmar is an acute-care hospital serving a population of approximately 150 000 in southeast Sweden. The population mainly consists of Swedish Caucasians with relatively few immigrants or subjects from other ethnic groups.

Clinical characterisation

The medical history of all subjects concerning diabetes, hypertension and current cigarette smoking was obtained from the patient or a relative following a written nurse-administrated standardized questionnaire. A history of hypertension was defined as the use of antihypertensive drugs and diabetes mellitus as the use of anti-diabetic drugs or diet treatment. Weight and height were measured with the subject in light clothing without shoes. Body mass index (BMI) was calculated as kilograms per m^2 . Blood pressure was measured with a mercury sphygmomanometer with the subject in the supine position.

Blood sampling procedures

At approximately 7 a.m. on the morning after admission to hospital, fasting venous blood samples were drawn to obtain DNA and for measurements of FFA, glucose, glycohaemoglobin (HbA1c), total cholesterol, LDL-cholesterol, HDL-cholesterol and triglycerides. Blood samples were obtained in tubes without additives and kept on ice for 30 minutes prior to centrifugation. Serum was immediately separated and kept at -70°C until analysis. Fasting serum FFA concentrations were measured using an acyl-CoA oxidase-based colorimetric kit (Wako Chemicals Inc., Richmond, VA, USA), on a Cobas Mira (Roche, Basel, Switzerland). Fasting plasma glucose was measured using a glucose dehydrogenase method, and fasting plasma total cholesterol, HDL-cholesterol, and triglyceride concentrations were analysed using commercially available kits using Cobas Integra 700 (Roche). Fasting plasma LDL-cholesterol concentration was calculated with the use of Friedewald's formula. HbA1c was measured using a high performance liquid chromatography (HPLC) method.^{22,23} The assays were performed according to the manufacturer's protocols.

Genotyping

The G to A nucleotide substitution in exon 2 of the FABP2 gene (A54T) was genotyped using an earlier described PCR-RFLP method⁵ with the following changes: exon 2 was PCR-amplified from 25 ng genomic DNA in a 20 μL volume consisting of 1 \times PCR buffer (Applied Biosystems, Roche, Basel, Switzerland), 0.25 mmol/L of each deoxy-NTP, 10 pmol of each primer, 2.5 mmol/L MgCl_2 , and 0.5 U *Taq* polymerase (Applied Biosystems, Roche). PCR reactions were initiated by denaturation (30 s at 94°C) followed by amplification for denaturation (30 s at 94°C), annealing (30 s at 55°C), and extension step (10 min at 72°C). The amplified 180 bp product was digested with *Hin6I* according to the manufacturer's instructions (Tamro Med-lab, Hisings Kärä, Sweden) and separated on a 3% agarose gel. The T54 allele lacking the *Hin6I* site migrated as a 180-bp fragment, whereas the A54 allele was cleaved and appeared as 99-bp and 81-bp fragments.

Ultrasonography, ECG and CT-scan

Duplex sonography combining B-mode imaging, colour flow and pulsed Doppler spectrum analysis was used (Sonos 5000 HP™ or Acuson Sequoia™ 512). A consultant clinical physiologist (MD) performed examinations using an 8 MHz probe with the subject in supine position. The Doppler angle was chosen as close to 60° as possible. Doppler measurements were made with the sampling volume in the common carotid artery (CCA) proximal to the bifurcation, in the internal carotid artery (ICA; where the highest peak velocity was chosen) and in the external carotid

artery (ECA). Maximum systolic peak ICA velocities above the normal limit 1.05 m/s^{24} were classified as stenosis of the ICA (ranging from 50% to 100%; degrees of stenosis less than 50% are not possible to detect with this method). Both the right and left carotid in each subject were investigated and the highest ICA-stenosis value of the two was used when the subjects were classified in either 1 = normal (<50% stenosis irrespective of the characteristics of a possible plaque; controls) or 2 = stenosis ($\geq 50\%$; cases). In addition, a 12-lead electrocardiogram (ECG) and a brain-computed tomography (CT) scan were made.

Data analysis and statistics

Variables not normally distributed (FFA, triglycerides and HbA1c) are presented as median \pm range, other continuous variables as means \pm SD, and categorical variables as percentages, calculated for each of the two groups (normal ICA velocities vs. ICA stenosis). Group differences were tested by Student's *t*-test (if normally distributed) or Mann-Whitney's U-test, or for frequencies, Fisher's exact test (2-tailed) using a commercially available computer program (STATISTICA version 6.0, StatSoft®, Tulsa, USA). *P* values ≤ 0.05 were considered statistically significant.

Logistic regression analysis was used to investigate associations between the dependent variable, ICA stenosis (yes/no) and the independent variables, FABP2 genotype (AA vs. TA + TT) together with possible risk factors for atherosclerosis, of which some were fixed in the model (see below). Both univariate and multivariate (stepwise, backwards) analysis were used (blood donors excluded). The following risk factors for atherosclerosis were fixed: Age (categorized in quartiles, for cut-off limits see Table 3), known diabetes (yes/no), measured systolic blood

pressure levels (categorized below or above the median value 160 mmHg) and smoking (yes/no). Other independent variables were gender (male/female), a history of hypertension (yes/no), diastolic blood pressure levels (below the median value of 84 mmHg or not), and use of oral lipid lowering medication (yes/no). Since our hypothesis is that atherosclerosis is partly caused by hyperlipidaemia, which in turn is associated with the FABP2 genotype, blood lipids were not included in the model.

Results

Differences between groups

Clinical characteristics of the patients are shown in Table 1 where they are classified according to ICA lumen. The group of patients with ICA stenosis were older, suffered from more diabetes and had lower concentrations of cholesterol and LDL-cholesterol than patients without ICA stenosis. There were no group differences in the proportions that smoked or had a history of earlier hypertension, nor were there any significant differences in systolic or diastolic blood pressure, BMI, fasting glucose, HbA1c, oral anti-diabetics or insulin.

Allele and genotype frequencies in patients with ICA stenosis did not deviate from those predicted by the Hardy-Weinberg equilibrium. Due to few patients with the TT genotype (only 2 patients with ICA-stenosis), the TT and TA genotypes were combined. Allele and genotype frequencies in the group of ICA stenosis (TT + TA, 68.0%; AA, 32.0%) did not differ significantly from the group of patients without ICA stenosis (TT + TA, 49.7%; AA, 50.3%; $p = 0.09$) (Table 2).

Table 1 Clinical characteristics of patients with and without internal carotid artery stenosis.

	Without ICA stenosis	ICA stenosis	<i>p</i> value
No. (male/female)	171 (107/64)	25 (18/7)	0.50
Age (years)	66.7 \pm 10.1	74.0 \pm 7.2	<0.01
Current smokers (%)	20.5	24.0	0.78
Earlier hypertension (%)	40.3	44.0	0.81
Systolic BP (mmHg)	155 \pm 26	167 \pm 29	0.05
Diastolic BP (mmHg)	84 \pm 11	90 \pm 15	0.07
Diabetes (%)	12.9	36.0	0.01
BMI (kg/m ²)	26.1 \pm 4.01	27.0 \pm 3.7	0.24
Lipid-lowering therapy (%)	6.4	4.0	0.56
Diabetics with oral anti-diabetics (%)	35.3	30.0	0.76
Diabetics with insulin (%)	34.4	20.0	0.39
F-glucose (mmol/L)	6.2 \pm 2.2	6.6 \pm 2.6	0.44
HbA1c (%)	4.7 (3.1–13.1)	5.0 (3.6–8.0)	0.18
F-Triglycerides (mmol/L)	1.4 (0.5–5.8)	1.5 (0.4–3.6)	0.62
F-cholesterol (mmol/L)	5.9 \pm 1.2	5.3 \pm 1.2	0.01
F-LDL (mmol/L)	3.9 \pm 1.1	3.3 \pm 1.2	0.02
F-HDL (mmol/L)	1.4 \pm 0.4	1.3 \pm 0.4	0.15
F-FFA (mmol/L)	0.57 (0.06–1.46)	0.63 (0.22–1.28)	0.60

Data are mean \pm SD, median and range or fraction (%).

Table 2 Genotype and allele frequencies in patients without ICA stenosis, with ICA stenosis and in healthy blood donors.

	Without ICA stenosis	ICA stenosis	Blood donors
FABP2 genotypes			
<i>n</i>	171	25	158
TT (<i>n</i> , %)	17 (9.9)	2 (8.0)	12 (7.6)
TA (<i>n</i> , %)	68 (39.8)	15 (60.0)	65 (41.1)
AA (<i>n</i> , %)	86 (50.3)	8 (32.0) ^a	81 (51.3) ^b
FABP2 alleles			
<i>n</i>	342	50	316
T (<i>n</i> , %)	102 (29.8)	19 (38.0)	89 (28.2)
A (<i>n</i> , %)	240 (70.2)	31 (62.0) ^c	227 (71.8)

^a $p = 0.09$, TT + TA vs. AA in patients with and without stenosis.

^b $p = 0.07$, TT + TA vs. AA in patients with stenosis and blood donors.

^c $p = 0.25$, T allele vs. A allele in patients with and without stenosis.

We also compared the genotype frequencies of the group of patients with ICA stenosis with the genotype frequencies in apparently healthy blood donors (TT + TA, 48.7%; AA, 51.3%). Neither in this group did genotype frequencies differ significantly from the group of patients with ICA stenosis (patients with ICA stenosis vs. blood donors, $p = 0.07$).

Association between FABP2 polymorphism and clinical characteristics

No associations between the different genotypes of the FABP2 gene polymorphism and blood pressure, BMI, fasting glucose, HbA1c and lipid concentrations were seen.

Association between ICA stenosis and FABP2 polymorphism in univariate and multivariate analysis

There were univariate statistically significant correlations between ICA stenosis and age ($p < 0.03$), a history of diabetes ($p < 0.01$), but not with the FABP2 TT + TA genotype ($p = 0.09$), blood pressure (systolic $p = 0.09$; diastolic $p = 0.29$; both categorized) or smoking ($p = 0.78$). Nevertheless, when known risk factors were fixed in the model (diabetes, blood pressure, age and smoking), the association between FABP2 genotype and carotid stenosis became statistically significant ($p = 0.04$) (Table 3). This was also true when using only statistically significant parameters in the model (diabetes, systolic blood pressure and FABP2; $p = 0.03$ for FABP2). Systolic and diastolic blood pressure (continuous or categorized) are strongly coupled and, therefore, only one of them could be incorporated in the model. Systolic blood pressure was chosen because it had a slightly stronger association with carotid stenosis than diastolic blood

Table 3 Multivariate logistic regression analysis for risk factors in ICA stenosis.

	Odds ratio	95% Confidence interval	<i>p</i> value
Diabetes mellitus			
no	1.00		
yes	4.93	1.76–13.8	<0.01
FABP2 genotype			
AA	1.00		
TT + TA	2.88	1.08–7.70	0.04
Systolic blood pressure			
<160 mmHg	1.00		
≥160 mmHg	2.51	0.91–6.95	0.08
Age			
<67 years	1.00		
67–74 years	1.57	0.98–2.52	
75–81 years	2.47	0.96–6.36	
>81 years	3.89	0.94–16.0	0.06
Current smoker			
no	1.00		
yes	2.27	0.74–7.0	0.15

pressure. Although lipids (LDL, HDL or triglycerides) were not included in the regression model for conceptual reasons (see methods) they had no influence on the significance in the association between FABP2 genotype and carotid stenosis.

Discussion

Stroke is a highly heterogeneous disease and the molecular basis of inherited stroke risk has been ill defined since different stroke subtypes have different genetic bases. Various genetic polymorphisms may also exert differential effects across different ages, sexes and ethnic groups.

To partly circumvent this problem, instead of studying the risk of stroke, we studied a so-called endophenotype or intermediated phenotype, i.e. ICA stenosis, in a group of patients with acute cerebrovascular disease. Thereby, we might enable differences in genotype frequencies between cases (patients with ICA stenosis) and controls (patients without ICA stenosis) to be detected. Known risk factors for ICA stenosis include age, male gender, hypertension, diabetes, and cigarette smoking.²⁵ ICA stenosis has also been associated with elevated triglycerides, low HDL and hypercholesterolemia.²⁶ Apart from the above-mentioned risk factors for carotid stenosis, variants in multiple genes have been associated with ICA stenosis. Among them are matrix metalloproteinase polymorphisms²⁷ as well as the Marburg I polymorphism of factor VII.²⁸ In addition, data from the San Antonio Family Heart Study indicates a substantial genetic influence in carotid artery plaque.²⁹

To our knowledge, this is the first direct study of the FABP2 (A54T) gene polymorphism in ICA stenosis. In favour of our hypothesis that the FABP2 gene could be considered a candidate gene polymorphism for ICA

stenosis, the gene polymorphism has been shown to affect the function of the protein *in vitro*.^{4,5} *In vivo*, the T54 allele has been associated with elevated plasma lipid levels. In the present study we did not find an association between the FABP2 T54 allele and significant fasting dyslipidaemia. Actually the patients with ICA stenosis did have lower total and LDL-cholesterol concentrations despite the fact that there were less than 10% on lipid lowering medication (statins), evenly distributed between the two groups. However, it is reasonable to believe that differences in lipid plasma concentration between different FABP2 genotypes are apparent, for the most part, in the postprandial state, but not in the fasting state.^{9,10}

The results of the study suggest that subjects with the T54 allele have more carotid atherosclerosis. Populations from different ethnic groups may have different genetic backgrounds and therefore different frequencies of genetic polymorphisms. A strength of the present study is the well-characterized study group, which came from a homogenous population (Caucasian), enrolled from only one hospital. Another strength is that we have studied a genetic variant that makes biological sense.

The results should, however, be interpreted with caution. Adequate sample size is important in genetic association studies. A limitation of the present study is the relatively small number of patients with carotid stenosis. A further limitation is that only current smoking (and not smoking in terms of current/former/never smoking) was assessed. In addition, the FABP2 genotype frequencies only reached statistical significance in the multivariate regression analysis, not in the univariate analysis.

Furthermore, the control group (patients without ICA stenosis) were slightly younger than the patients with ICA stenosis. If the FABP2 A54T polymorphism is associated with the development of atherosclerosis, the frequency of the T54 allele may be higher in the present control group selected from patients with stroke than in an age-matched control group chosen from a healthier population. In order to partly circumvent this problem, we therefore compared the patients with ICA stenosis with a second control group, blood donors. Here we found a similar association with a nonsignificant trend towards a lower frequency of the T54 allele in the blood donors compared with the patients with ICA stenosis. The group of blood donors had not been assessed with an ultrasound of the carotids and the group was younger than the patients with ICA stenosis, but it is reasonable to assume that the frequency of ICA stenosis in this group will still be relatively low when the group has reached the same age as the group of patients with ICA stenosis, strengthening our hypothesis. In order to decide whether our results are likely to represent a true effect rather than statistical noise, an independent replication study in another population is needed.

In summary, our findings suggest a possible involvement of the FABP2 T54 allele in the pathogenesis of ICA stenosis.

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