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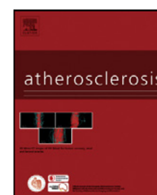

Osteopontin gene variation and cardio/cerebrovascular disease phenotypes

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ABSTRACT

We aimed at associating common osteopontin (OPN) gene variants with cardiovascular disease phenotypes.We scanned the OPN gene in 190 chromosomes from myocardial infarction (MI) patients and identified five variants in the promoter, three synonymous and one non-synonymous variant. All variants were investigated in case–control studies for MI (ECTIM: 990 cases, 900 controls) and brain infarction (BI) (GÉNIC: 466 cases, 444 controls). Promoter variants were functionally analyzed by bandshift assays, the coding D147D [T/C] by Western blot. Allele D147D C was independently and significantly associated with lower apoB levels ($P = 0.044$ [ECTIM] $P = 0.03$ [GENIC]), its allele frequency was significantly lower in patients with BI compared to controls (OR [95% CI] 0.39 [0.20–0.74], $P = 0.004$), and C allele carriers had a significantly lower frequency of presence of carotid plaques ($P = 0.02$). Bandshifts with HepG2 and Ea.hy926 nuclear proteins did not reveal any functionality of promoter variants, whereas the OPN-441C-containing construct resulted in reduced OPN protein expression in Western blots, complying with its potential protective effect on the phenotypes studied.We here provide evidence that a portion of the OPN locus is likely to associate with cardiovascular disease-related phenotypes. However, further experiments are warranted to clarify the functional role of OPN variants.

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1. Introduction

Osteopontin (OPN, OMIM *166490) is a phosphorylated matrix glycoprotein that contains an Arg–Gly–Asp (RGD) cell attachment sequence [1] and promotes adhesion and migration of vascular smooth muscle cells (VSMCs) and endothelial cells in vitro [2]. With respect to the cardiovascular system and via distinct receptors [3], OPN participates in SMC, endothelial cell and leukocyte adhesion/migration within vascular injury sites [4], elevated OPN mRNA and protein expression has been demonstrated in human aortic, coronary and carotid atherosclerotic plaques [5], mostly accompanied with significant calcification. As SMCs and endothelial cells express OPN at very low levels, the major source of OPN in atherosclerotic plaques was reported to be infiltrating macrophages [6]. Transgenic mice overexpressing OPN display medial thickening and neointimal formation [7], and OPN-deficiency attenuates atherosclerosis, but associates with vascular calcification [8].

The human OPN gene is located on chromosome 4q21–q25, spans about 11 kb, and consists of seven exons; the protein is composed of 314 amino acid residues, and splice variants have been described [9]. The aim of the present study was to

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determine whether OPN gene variants are associated with cardio/cerebrovascular disease phenotypes. We, therefore, scanned the 5′-flanking region, the entire coding region, exon/intron boundaries, and the 3′-region of the OPN gene for genetic polymorphisms by use of polymerase chain reaction (PCR)/single-strand conformation polymorphism (SSCP) analysis and investigated all identified polymorphisms in case–control studies for myocardial infarction (MI) (ECTIM: Etude Cas-Temoins sur l'Infarctus du Myocarde, the ECTIM Study, 990 cases and 900 controls) and brain infarction (BI) (GÉNIC: Étude du Profil Génétique de l'Infarctus Cérébral, the GÉNIC Study, 466 cases and 444 controls). In addition, we investigated the in vitro functionality of identified single-nucleotide polymorphisms (SNPs) where appropriate.

2. Materials and methods

2.1. The ECTIM Study

ECTIM is a study of patients with MI from regions covered by the World Health Organization's Monitoring Trends and Determinants in Cardiovascular Disease (MONICA) registers and of control subjects, representative of each geographic area in United Kingdom and France. Informed written consent was obtained from all participants and their family doctors. A detailed description is presented in the online-only Data Supplement.

2.2. The GÉNIC Study

The GÉNIC Study, of case–control design, examined genetic susceptibility for BI and involved 12 French neurological departments. The research protocol was approved by the ethics committee of Hôpital Cochin and all subjects signed informed consents. A detailed description is presented in the online-only Data Supplement.

2.3. Carotid Ultrasonography Studies in the GÉNIC Study

All subjects had carotid ultrasound examination evaluating the presence and site of plaques. A detailed description is presented in the online-only Data Supplement.

2.4. Screening of OPN gene for polymorphisms and genotyping

Genomic DNA was prepared from white blood cells by using either phenol–chloroform extraction or a salting out method [10]. Based on published OPN sequences (Acc. D18413), overlapping fragments of <320 bp from 95 high-risk MI patients (190 alleles) from the ECTIM Study covering 2314 bp of the 5′-flanking region, 109 bp of 5′UTR, the entire coding region, including exon/intron boundaries, and 165 of the 3′-region were amplified. A detailed description is presented in the online-only Data Supplement.

2.5. Direct sequencing of the microsomal triglyceride transfer protein (MTP) gene

From the published sequences of the MTP gene [11], 4640 bp, including the 5′-flanking, coding, intronic (exon–intron boundaries), and 3′-flanking regions were directly sequenced (ABI PRISM 377, PerkinElmer, Rodgau-Jügesheim, Germany) in 11 individuals with the lowest apoB (0.54–0.67 g/L) and 14 with the highest apoB levels (2.56–4.76 g/L) from the ECTIM Study. Specific amplification protocols can be obtained online (http://genecanvas.ecgene.net).

2.6. Statistical analysis

Data were analyzed using the SAS statistical software. Significance level was $P < 0.05$.

2.7. Data analysis in the ECTIM and GÉNIC Studies

Allelic frequencies were calculated by gene-counting. For both studies, Hardy–Weinberg equilibrium was tested with the Chi-square test with 1 degree of freedom separately in cases and controls from each recruitment center. We compared genotype distributions in cases and controls and computed ORs using logistic regression analysis with adjustment for age and sex. In ECTIM, for case/control comparison, controls with CAD were excluded. Linkage disequilibrium (LD) and haplotype analyses were performed using the THESIAS program [12] available on line (http://genecanvas.ecgene.net). Differences in haplotypic frequencies between cases and controls were tested by means of the likelihood-ratio test. Odds ratio (OR; 95% CI) compared cases with and without a history of angina pectoris, as well as controls with and without the presence of hypertension (defined as a systolic blood pressure >160 or diastolic >95), associated with −616 TG + GG vs. −616 TT. Differences in apoB levels according to genotypes 147 TC + CC vs. 147 TT were tested by a general linear model adjusted for age, sex and center. Plasma apoB level was log-transformed for tests because of positive skewness, but untransformed values are presented in the table.

"In GÉNIC, the main analysis regarding the association of OPN D147D with BI covered the entire study group. Further analyses were stratified according to the four main BI subtypes (atherothrombotic, lacunar, cardioembolic strokes, and strokes of unknown cause). Analyses concerning strokes of undetermined cause are not reported, because this group is too heterogeneous. Analyses restricted to cases and controls, both free of previous cardiovascular or cerebrovascular history (i.e., incident cases), are also reported. The homogeneity of the association between the OPN D147D and BI across gender and age groups was tested by introducing multiplicative terms into the logistic regression models.

We studied the association between the D147D and several characteristics (traditional cardiovascular risk factors [hypertension, diabetes mellitus, smoking, cardiovascular history and lipid profiles other than apoB], apoB level and carotid atherosclerosis [IMT and plaques]) in cases and controls considered together using logistic regression (dichotomous variables) or analysis of covariance (continuous variables) adjusted on age, sex and case–control status. Analyses concerning lipid parameters were adjusted for three variables that served as confounders (use of lipid-lowering drugs, blood sample delay, and degree of disability estimated by the Rankin scale), and analyses concerning carotid atherosclerosis were adjusted for the center effect. Finally, to assess the independent contribution of OPN D147D on BI risk, we used a backward selection procedure with a significance level of 0.10 for staying in the model; traditional cardiovascular risk factors, apoB level and carotid atherosclerosis were included as candidate variables, and age, sex as forced variables".

2.8. In silico analyses of putative transcription factor binding sites (TFBS)

Variants in the regulatory region of the OPN gene were subjected to computer-aided analyses using the Alibaba2.1/Transfac7 net-based search tool (http://www.gene-regulation.com). Pairsim and minimal matrix conditions as well as factor type were adjusted appropriately.

2.9. Cell culture

Detailed protocols on the human embryonic kidney cell line HEK293T, the human osteogenic sarcoma cell line SaOs-2, the human hepatoma cell line HepG2, the endothelial cell line EA.hy 926 as well as the human monocytic cells THP-1 are presented in the online-only Data Supplement.

2.10. RNA extraction and generation of cDNA

Total RNA from cells was harvested by the RNeasy method (Qiagen, Hilden, Germany) following the manufacturer's instructions. A detailed description is presented in the online-only Data Supplement.

2.11. Generation of eukaryotic OPN expression vectors pOPN-441T/CMV and pOPN-441C/CMV

Full-length wildtype (wt) OPN cDNA was amplified by PCR (GoTaq, Promega, Mannheim, Germany) using primers 5′-GGAAGCTTACTACCATGAGAATTGCAGTG-3′ (sense; harbouring a *Hind*III restriction site; underlined) and 5′-GGCGGCCGCTTTC-TCCTTTTAATTGACCTC-3′ (anti-sense; harbouring a *Not*I restriction site; underlined) and cDNA from cAMP-stimulated HepG2 cells as template. A detailed description is presented in the online-only Data Supplement.

2.12. Transient transfection of HEK293T cells

HEK293T cells were transfected with Lipofectamine 2000 (Invitrogen). A detailed description is presented in the online-only Data Supplement.

2.13. Extraction of crude protein extracts and Western blot

HEK293T cells were transiently transfected with expression vectors pRC/CMV as mock control and both pOPN-441T/CMV and pOPN-441C/CMV. A detailed description is presented in the online-only Data Supplement.

2.14. Extraction of nuclear proteins

A detailed description of how nuclear proteins from HepG2, EA.hy 926, differentiated and undifferentiated SaOs-2 and THP-1 were harvested is presented in the online-only Data Supplement.

2.15. TdT-labeling and annealing

All oligonucleotides (Supplementary Table 1) were synthesized at a coupling efficiency of >98.5% and purified twice by HPLC (IBA, Goettingen, Germany). A detailed description is presented in the online-only Data Supplement.

2.16. Electrophoretic mobility shift assay (EMSA)

EMSAs were performed using the LightShift Chemiluminescent EMSA Kit (ThermoFisher). A detailed description is presented in the online-only Data Supplement.

3. Results

3.1. Identification of polymorphisms in the OPN gene

In the OPN gene, a total of nine variants were identified; five in the 5′-flanking region (G-655T, G-616T, T-443C, DelG-156InsG, T-66G), three synonymous variants in exon 6 (D94D [T/C] rs4754, D147D [T/C]) and exon 7 (A250A [T/C] rs1126616); completely associated with D94D), and one non-synonymous variant in exon 6 (E122K [G/A]). The complete description of the polymorphisms and the pairwise LD coefficients are available at our website (http://genecanvas.ecgene.net).

3.2. Identification of polymorphisms in the MTP gene

Direct sequencing of the MTP gene in 50 chromosomes of ECTIM participants led to the identification of 31 variants (Supplementary Table 2), which are all listed online-only Data Supplement.

3.3. Results in the ECTIM and GÉNIC Studies

3.3.1. The ECTIM Study
In the ECTIM Study, we identified only one individual carrying G-655T and four individuals carrying E122K. Globally, genotype and allele frequencies of the different polymorphisms did not significantly differ between patients and controls from the ECTIM Study (Table 1). In the haplotype analysis, due to the rare occurrence of two variants, only six haplotypes with frequency >0.01 were estimated (data not shown). In particular, G^{-616} and C^{147} were each carried on a single haplotype [G^{-616}-T^{-443}-T^{94}-T^{147} and T^{-616}-T^{-443}-T^{94}-C^{147}], respectively. There was no significant difference

Table 1
Distribution of OPN genotypes and alleles in patients with myocardial infarction and controls in the ECTIM Study.

Position	Status	Genotype, *n* (%)			P[a]	Allele 2
		11[b]	12[b]	22[b]		Frequency
T-616G	Cases Controls	890 (90.9) 810 (90.5)	84 (8.6) 82 (9.2)	5 (0.5) 3 (0.3)	P = 0.76	0.048 0.049
T-443C	Cases Controls	245 (25.2) 231 (26.0)	512 (52.7) 422 (47.5)	215 (22.1) 235 (26.5)	P = 0.046	0.485 0.502
D94D	Cases Controls	571 (57.8) 497 (55.2)	346 (35.0) 346 (38.4)	71 (7.2) 58 (6.4)	P = 0.31	0.247 0.256
E122K	Cases Controls	986 (99.8) 896 (99.6)	2 (0.2) 4 (0.4)	0 (0.0) 0 (0.0)	P = 0.35	0.001 0.002
D147D	Cases Controls	957 (96.9) 870 (96.4)	31 (3.1) 32 (3.5)	0 (0.0) 1 (0.1)	P = 0.52	0.016 0.019

Since polymorphisms D94D and A250A were in complete LD, genotype and allele frequencies are only given for D94D.
[a] Cases vs. corresponding controls.
[b] The frequent allele is coded 1 and the minor allele is coded 2.

Table 2
Mean apoB levels (g/L) according to OPN D147D T/C in cases and controls from the UK and France (ECTIM Study).

OPN D147D genotype	ApoB (g/L) mean ± S.E.M.	
	Cases n = 965	Controls n = 931
TT	1.43 (0.01)	1.34 (0.01)
TC + CC	1.29 (0.07)	1.30 (0.06)

Difference between TT and C+ effect on apoB level, cases and controls pooled: P = 0.044. Test of homogeneity of OPN D147D effect between cases and controls: P = 0.23.

between cases and controls in the haplotype frequencies (global test: P = 0.87).

The D147D C allele was significantly associated with lower apoB levels, the difference between T homozygotes and C carriers, for cases and controls pooled, being P = 0.044 (test of homogeneity of OPN D147D effect in cases and controls: P = 0.23; Table 2). Moreover, this effect was observed in the three centers and in both sexes (P = 0.94 and 0.83, respectively, for interaction, data not shown).

In addition, in cases carrying the minor allele of T-616G, a lower frequency of angina pectoris was observed (OR [95% CI] 0.45 [0.27–0.90] P = 0.022), this effect being observed across all centers and sexes (data not shown). Similarly, in controls, across centers and for both sexes, there was a significantly lower frequency of hypertension in −616G carriers (OR [95% CI] 0.54 [0.32–0.93], P = 0.025) (data not shown).

3.3.2. The GÉNIC Study

In accordance with the ECTIM Study, significant genotype–phenotype associations were observed for OPN D147D. Distributions of D147D genotypes and allele frequencies in cases and controls are shown in Table 3, overall, and according to sex, and age. Overall, the frequency of the D147D T/C genotype was significantly lower in cases compared to controls (OR [95% CI] 0.39 [0.20–0.74], P = 0.004), suggesting a protective association of OPN D147D with BI. Similar results were obtained in analyses restricted to cases and controls free of previous cardiovascular or cerebrovascular history (Table 3). There was no evidence of heterogeneity in this association across sex (P = 0.54) and age groups (P = 0.79). For each of the main subtypes, the frequency of TC carriers was lower in cases than in controls, although not significantly due to inadequate statistical power given the small number of TC heterozygotes (Table 3).

In accordance with the ECTIM Study, TC carriers had a lower plasma apoB level than TT carriers in cases (mean ± S.E.M., 1.07 ± 0.1 vs. 0.94 ± 0.08) as well as in controls (mean ± S.E.M., 0.94 ± 0.01 vs. 0.86 ± 0.04). The association between TC carriers and apoB was significant in multivariate analysis performed on cases and controls considered together (Table 4). Among others characteristics tested, TC carriage was only associated with a lower prevalence of carotid plaques (P = 0.02). Both carotid plaques and apoB remained independently associated with C alleles in multivariate analysis adjusted on case–control status, lipid-lowering drugs, blood sample delay, degree of disability, and center.

To assess the independent contribution of OPN D147D on the BI risk, OPN D147D, all traditional risk factors, apoB, and markers of atherosclerosis were implemented into a multivariate logistic regression analysis. Using a backwards selection procedure, OPN D147D was removed with a P-value of 0.15 (OR [95% CI] 0.58 [0.27–1.22]) (Table 5). When marker of carotid atherosclerosis was not considered as candidate variables, OPN D147D remained associated with BI independently of traditional cardiovascular risk factors (OR [95%CI] 0.45 [0.22–0.91], P = 0.03).

Table 3
Distribution of OPN D147D T/C genotypes in cases with BI and controls (GÉNIC Study).

OPN D147D T/C	n	TT, n (%)	TC, n (%)	Freq. C %	OR (95% CI)[a]	P[a]	Cases and controls free of cardio and cerebrovascular history		
					TC vs. TT		n[b]	OR (95% CI)[a] TC vs. TT	P[a]
Controls[c]	469	435 (92.8)	34 (7.2)	0.036	1.00 (reference)	–	414	1.00 (reference)	–
All cases	443	430 (97.1)	13 (2.9)	0.015	0.39 (0.20–0.74)	0.004	286	0.30 (0.13–0.70)	0.005
Atheromthrombotic	100	99 (99.0)	1 (1.0)	0.005	0.14 (0.02–1.00)	0.05	57	–	–
Lacunar	94	89 (94.7)	5 (5.3)	0.027	0.70 (0.27–1.84)	0.47	64	0.78 (0.27–2.29)	0.65
Cardioembolic	69	68 (98.5)	1 (1.5)	0.007	0.17 (002–1.31)	0.09	48	0.22 (0.03–1.69)	0.15
Unknown cause	102	98 (96.1)	4 (3.9)	0.020	0.54 (0.19–1.57)	0.26	69	0.38 (0.09–1.65)	0.20
Women									
Controls	180	166 (92.2)	14 (7.8)	0.039	1.00 (reference)	–	168	1.00 (reference)	–
Cases	166	162 (97.6)	4 (2.4)	0.012	0.29 (0.09–0.91)	0.03	116	0.20 (0.04–0.90)	0.04
Men									
Controls	289	269 (93.1)	20 (6.9)	0.035	1.00 (reference)	–	246	1.00 (reference)	–
Cases	277	268 (96.7)	9 (3.3)	0.016	0.45 (0.20–1.00)	0.05	170	0.39 (0.14–1.06)	0.06
<68 years[d]									
Controls	231	216 (93.5)	15 (6.5)	0.032	1.00 (reference)	–	212	1.00 (reference)	–
Cases	209	203 (97.1)	6 (2.9)	0.014	0.43 (0.16–1.11)	0.08	151	0.42 (0.13–1.31)	0.13
≥68 years[d]									
Controls	238	219 (92.0)	19 (8.0)	0.040	1.00 (reference)	–	202	1.00 (reference)	–
Cases	234	227 (97.0)	7 (3.0)	0.015	0.36 (0.15–0.87)	0.02	135	0.22 (0.06–0.76)	0.02

[a] ORs were computed using logistic regression, with adjustment for age and sex.
[b] Number of subjects without previous cardio or cerebrovascular history.
[c] Test for Hardy–Weinberg equilibrium, P = 0.99.
[d] Median age.

Table 4
Association between OPN D147D T/C and several characteristics in the GÉNIC Study.

OPN D147D	Cases		Controls		P[a]
	TT $n = 430$	TC $n = 13$	TT $n = 435$	TC $n = 34$	
ApoB (g/L), mean (S.E.M.)	1.07 (0.01)	0.94 (0.08)	0.94 (0.01)	0.86 (0.04)	0.03
Total cholesterol (g/L), mean (S.E.M.)	2.02 (0.02)	1.90 (0.09)	1.82 (0.02)	1.76 (0.04)	0.29
Triglyceride (g/L), mean (S.E.M.)	1.54 (0.04)	1.24 (0.14)	1.38 (0.03)	1.32 (0.11)	0.12
Carotid plaques, n (%)	276 (67.2)	6 (50.0)	178 (43.0)	10 (33.3)	0.02
Carotid IMT (mm), mean (S.E.M.)	0.80 (0.01)	0.77 (0.03)	0.74 (0.01)	0.75 (0.03)	0.63

[a] Logistic regression was used for categorical variables, and analyses of covariance for continuous variables, both adjusted on age, gender and cases–control status. Analyses concerning lipid parameters were further adjusted for use of drugs, blood sample delay, and degree of disability. Analyses concerning the marker of carotid atherosclerosis (plaques and IMT) were adjusted for a center effect.

Table 5
Multivariable backward logistic regression analysis of risk factors for BI.

Candidate variables	Step	OR (95%CI)	P-value
Factors removed			
Diabetes	1	1.30 (0.81–2.09)	0.27
Triglycerides[a]	2	0.88 (0.71–1.09)	0.25
ApoB[a]	3	1.10 (0.85–1.42)	0.47
OPN D147D	4	0.58 (0.27–1.22)	0.15
Factors staying in the models			
Hypertension	4	1.43 (1.02–1.99)	0.04
Current smoker	4	1.54 (1.04–2.29)	0.03
Cardiovascular history	4	1.63 (1.06–2.53)	0.03
Total cholesterol[a]	4	1.76 (1.48–2.09)	<0.001
Carotid IMT[a]	4	1.35 (1.18–1.55)	<0.001
Carotid plaques	4	2.89 (2.04–4.11)	<0.001

[a] OR calculated per one standard deviation increase. The backward multiple logistic model was performed in 791 subjects (395 cases and 396 controls) with complete information on candidate and forced variables (i.e. age and sex).

3.4. *Genetic variants within the OPN regulatory region fail to interact with nuclear proteins*

An in-depth in silico analysis of a 30-bp DNA sequence flanking the genetic variants within the OPN regulatory region using the net-based program Alibaba2.1 and the Transfac 7.0 data base predicted that neither variant resides within a putative TFBS. A detailed description of results is presented in the online-only Data Supplement.

3.5. *The D147D polymorphism alters the efficiency of OPN gene expression at the protein level*

HEK293T cells did neither endogenously express OPN mRNA, nor immunoreactive OPN was detectable. When transiently transfected with expression vectors for both OPN alleles, cells revealed a lower expression of OPN-441C compared to OPN-441T, which would be in accordance with the presumed protective role of this allele (Fig. 1).

4. Discussion

In our present analysis, the OPN D147D C allele was (I) more frequent in controls than in patients with BI, (II) associated with a reduced frequency of carotid plaques in patients and controls from GENIC; in both the ECTIM and GÉNIC Studies, (III) independently associated with lower plasma apoB levels compared to non-carriers, and (IV) and the OPN-441C construct revealed a slightly lower expression compared to OPN-441T in our Western blot analysis. If the D147D C allele was actually protective, its frequency should increase with age. Indeed, the C allele was significantly more frequent in GENIC controls, than in ECTIM controls, GENIC controls being in average 9 years older than those in ECTIM. As other obesity-related phenotypes [13] or clinical outcome in patients with stable CAD [14] have been associated with plasma OPN levels, these phenotypes should be related in future association studies with OPN genetic variation.

With respect to the link between OPN and plasma apoB levels, Strom et al. [15] reported significantly decreased total plasma cholesterol levels in apoE/LDL receptor/OPN triple knockout compared to apoE/LDL receptor-deficient mice. Other cytokines such as IL-1 and TNF-α have been shown to decrease apoB secretion in human fetal hepatocytes by decreasing MTP large subunit mRNA and protein expression in HepG2 cells. However, to the best of our knowledge, there is no obvious pathophysiological link between OPN and apoB generation, secretion or degradation.

Alternatively, OPN D147D might be in LD with a functional variant in another gene controlling apoB regulation as for example the MTP, which resides on chromosome 4q22–q24 close to the OPN locus 4q21–q25. Indeed, MTP gene mutations cause abetalipoproteinemia (Bassen–Kornzweig syndrome), a rare autosomal recessive disease characterised by the virtual absence of plasma apoB containing lipoproteins due to a truncated MTP protein [16]. Although both genes are separated by approximately 13 million bp, we hypothesized that LD of OPN D147D with a functional MTP variant may explain the association with apoB levels. A subsequent analysis of OPN D147D with formerly typed MTP variants A-400T (rs1800803) and T-164C (rs1800804) in the ECTIM Study resulted in a relatively low although significant LD coefficient. However, an in-depth analysis of the genomic region encompassing

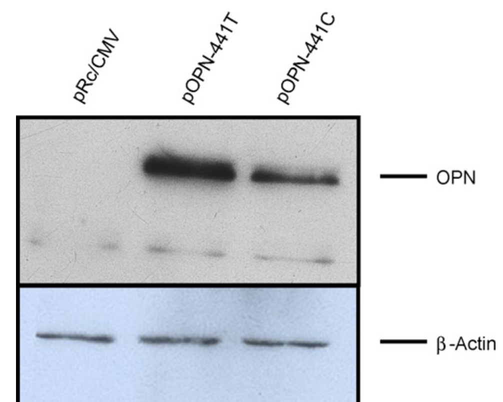

Fig. 1. Detection of OPN protein in transiently transfected HEK293T cells. Western blot analyses of overexpressed OPN protein from expression vectors bearing either the 441T or 441C allele. Proteins were extracted 24 h after transfection. OPN-negative HEK293T cells, transiently transfected with expression vectors for both OPN alleles, revealed a lower expression of OPN-441C (upper panel). Mock transfected cells do not express OPN (pRC/CMV; upper panel, left lane). Even loading was ascertained by β-actin immunodetection (bottom panel).

OPN and MTP using the HAPMAP data (http://www.hapmap.org) on the Caucasian CEU population data showed that additional genes reside between OPN and MTP separated by multiple recombination spots (data not shown), making an indirect association of OPN through LD with MTP an unlikely event. This observation is further corroborated by the fact that neither of the MTP variants was associated with any lipid profile in the ECTIM Study [17].

As D147D is a synonymous SNP, it does not alter the amino acid composition of OPN. However, synonymous variants could potentially affect mRNA splicing or stability [18] or even translation efficiency giving rise to an altered protein folding [19]. Even if were able to demonstrate that transiently transfected OPN-negative HEK293T cells revealed a lower expression of OPN-441C compared to OPN-441T, the precise mechanisms, explaining this functional aspect, remain to be demonstrated in appropriate further experiments.

In our recently performed large-scale analysis in 470 BI patients from GENIC with measured CCA-IMT [20], we analyzed 54 polymorphisms originating from 40 different genes with suspected roles in atherosclerosis and observed that OPN T-443C was associated with increased CCA-IMT.

Giacopelli et al. [21] performed promoter experiments with OPN variants including T-443C in different cell lines and reported that the haplotype carrying the less frequent alleles at the polymorphic sites conferred a significantly reduced level of reporter gene expression; in their study −443T was the minor allele. The authors proposed that OPN T-66G modified the binding affinity for the SP1/SP3 transcription factors.

This is at odds with our results, since in the present study we were unable to identify any DNA–protein interactions in EMSA experiments with nuclear extracts from different endogenously expressing cell lines (differentiated THP-1 macrophages, undifferentiated and differentiated SaOs-2, HepG2, and EA.hy 926) and for the sequence portions flanking the polymorphic sites at positions −443 and −616. The DNA samples were 30 bp in length (coupling efficiencies of >98.5%), evenly flanking the variant site and thus ensuring (a) sufficient sequence to allow specific TFBS interaction and (b) short enough to assign any differential binding pattern to the presence of the SNP alone. However, with respect to the report by Giacopelli et al. [21], they did not present picture proof of the appropriate bandshift results and did not specify their results with individual cell lines, making it difficult to finally follow their results. Our in silico analyses of the respective polymorphic sites using the net-based search algorithm Alibaba2.1 also failed to confirm the reported putative MYT1-site. TFBS are composed of a DNA sequence in which each position is of different importance and relevance for the molecular function of the entire site. The reliability of a computerized TFBS search strictly depends upon the exactness with which each position therein can be assigned. This, in turn, also depends upon the number of such sites which have been reported to the underlying database as truly functional in vitro or in vivo. It is, therefore, not unusual when computerized analyses of an identical DNA sequence reveal different results given the underlying TFBS databases have changed. In most cases, a better data situation results in a more reliable omission of “false positive” hits and formerly predicted sites can be safely excluded; the Transfac 7.0 database differs from former versions by several thousand new entries.

In conclusion, in the present analysis, but also taking earlier results into account, we show that a portion of the OPN locus is likely to be associated with cardio/cerebrovascular disease-related phenotypes. Even when Western blot analyses showed a slightly decreased expression of the pOPN-441C carrying construct, which would comply with its protective effect on the phenotypes studied, further functional experiments are necessary to support this notion.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.atherosclerosis.2009.02.015.

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