RESEARCH ARTICLE

Genetic polymorphisms of *T-1131C APOA5* and *ALOX5AP SG13S114* with the susceptibility of ischaemic stroke in Morocco

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Abstract

Ischaemic stroke is a multifactorial disease. Genetic polymorphisms involved in lipid, inflammatory and thrombotic metabolisms play an important role in the development of ischaemic stroke. The present study aimed to assess the relationship between *T1131C APOA5* and *SG13S114 ALOX5AP* polymorphisms and the risk of ischemic stroke in 175 cases and 201 controls. Genotyping was performed by high resolution melting and polymerase chain reaction restriction fragment length polymorphism methods. In the case of *T-1131C APOA5*, a modest risk of ischaemic stroke was noticed with CC (OR: 2.86; 95% CI = 1.24–6.58; Pc = 0.039) and C allele (OR: 1.54; 95% CI = 1.01–2.33; Pc = 0.014). For *SG13S114 ALOX5AP*, a significant association was observed among subjects with TT (OR: 2.57; 95% CI =1.49–4.83; Pc = 0.009) and T allele (OR: 1.59; 95% CI = 1.16–2.19; Pc = 0.008). According to the risk factors of ischaemic stroke, a positive correlation was observed only between *SG13S114* variant of *ALOX5AP* gene and hypertension (Pc = 0.026). Despite lower sample size, *T-1131C APOA5* and *SG13S114* variants could be considered an independent genetic risk factor of ischaemic stroke in Moroccan population.

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Introduction

After cardiovascular diseases and malignant tumours, stroke is the third leading cause of death in developed countries. Both males and females can be affected by this disease at any time of life. Ischaemic stroke (IS) which represents 80% of all cases of strokes is a multifactorial disease depending on several mechanisms (Bonita *et al.* 2004; Walt 2004). According to the results of an epidemiological investigation, IS affects 4.1% of the Moroccan population (International Symposium on Stroke, Rabat, Morocco). In addition to traditional risk factors for IS such as diabetes, tobacco, alcohol and oral contraception, genetic factors have also been implicated in the pathogenesis of many local cerebral dysfunctions. It is known that genes involved in lipid metabolism (e.g. apolipoprotein A5, *APOA5*; Havasi *et al.* 2006), inflammation (e.g. arachidonate 5-lipoxygenase-activating protein *ALOX5AP* and endothelial nitric oxide synthase) (Helgadottir *et al.* 2004; Lõhmussaar *et al.* 2005; Diakite *et al.* 2014), and in coagulation system (Hamzi *et al.* 2011; Diakite *et al.* 2015) play a significant role in the development of IS. Thus, these mechanisms may contribute alone or in synergy with other genes or factors external to the development of IS process. Many studies have investigated the associations of *APOA5* and *ALOX5AP* polymorphisms with increased risk for IS. However, in many previous reports on IS, contradictory associations have been observed with these polymorphisms. *T-1131C APOA5* located in the promoter region of the *APOA5* gene (Pennacchio *et al.* 2002) has been associated with a high risk (Jaromi *et al.* 2010; Pi *et al.* 2012). The *SG13S114* variant of *ALOX5AP* gene located on chromosome 13q12 conferred a risk of IS in some populations (Helgadottir *et al.* 2004). However, this risk was not found in other populations (Zintzaras and Lau 2008).

Thus, based on the assumption that *T-1131C APOA5* and *SG13S114* variants could be associated with the risk of IS in Moroccon population, we carried out the present study with the aim to assess the association of these two polymorphisms with IS.

Materials and methods

Study population

The present case–control study comprises 175 IS patients (average age, 57.1 ± 2) recruited at the Neurology Services,

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Keywords. *T-1131C APOA5*; *ALOX5AP SG13S114*; susceptibility; ischaemic stroke.

Neurology Hospital University Center (CHU) Ibn Rochd of Casablanca and Rabat between March 2009 and February 2013, and 201 controls (average age, 54.6.±2) healthy blood donors without any history of IS and cognitive disorders. Demographic data (age and sex) and risk factors of cerebral ischaemia such as hypertension, diabetes, smoking and alcohol consumption were collected from medical records. After cardiovascular and neurological examinations, a computer tomography or magnetic imaging resonance (MRI) was performed for each patient. According to the classification of trial of ORG 10172 in acute stroke treatments (TOAST), IS patients were divided into four groups, atherosclerosis (diseases of the large arteries with an infarct diameter >1.5 cm), lacunar (occlusive disease perforating small arteries with infarct diameter <1.5 cm), cardio embolic and other causes. All participants accepted and signed the informed consent. The present study was approved by the local Ethics Committee Hassan II University, Casablanca, Morocco.

DNA extraction

Five millilitres of peripheral blood were collected from each patient and control in an EDTA tube. Genomic DNA was isolated from white blood cells using salting out method. The spectrophotometer was used to determine the quality and quantity of the DNA. Two essays were performed, 100 ng/μL DNA for amplification by polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP) and 20 ng/μL to high resolution melting (HRM).

Genotyping by HRM amplification

The different genotypes of *ALOX5AP SG13S114* were determined by HRM 7500 Fast Real-Time PCR system (AB Applied Biosystems, Foster City, USA). The MeltDoctor^{TM} HRM Master Mix which contains SYTO9 as the DNA intercalating dye was used for the protocol of HRM reaction. The forward and reverse primers of *SG13S114* were previously described by Zhang *et al.* (2006). The amplicon size of this variant was 212 bp. According to the manual HRM Applied Biosystems, a final reaction volume of 20 μL was used for the amplification product HRM (Graham *et al.* 2005; Price *et al.* 2007; Reed *et al.* 2007). HRM amplification conditions were: 10 min of enzyme activation at 95°C (holding stage), followed by 40 cycles of 15 s at 95°C and 1 min at 60°C, and finally followed by melting curve phase: 10 s of denaturation at 95°C, 1 min of hybridization at 60°C, 15 s of high resolution melting at 95°C and 15 s of final hybridization at 60°C. Using Fast System SDS 7500 v2.0.1 software analysis of the results was performed. The interpretation of AA, AT and TT genotypes of *SG13S114* was performed from the temperature curves in reference to the curves of the contracts. After interpreting the results by HRM, the plates containing the amplification products were stored at 4°C and digested with the restriction enzyme *Ase*I to confirm the results.

Genotyping by PCR-RLFP

The *T-1131C* polymorphism of APOA5 was identified by PCR-RFLP as described by Talmud *et al.* (2002). The PCR conditions of the *T-1131C APOA5* variant were: 2 min of initial denaturation at 96°C, followed by 35 cycles of 30 s at 95°C, 30 s at 55°C, 30 s at 72°C and final extension of 5 min at 72°C. After digestion with *Mse*I restriction enzyme, PCR products revealed three fragments (267, 109 and 22 bp) for TT wild type, four fragments (289, 267, 109 and 22 bp) for TC and two fragments (289 and 109 bp) for CC. The genotypes for *ALOX5AP SG13S114* were detected by PCR-RFLP as described by Zhang *et al.* (2006). The PCR conditions were: 10 min of initial denaturation at 95°C, followed by 35 cycles of 30 s at 95°C, 30 s at 61°C, 40 s at 72°C and final extension of 10 min at 72°C. PCR products were digested with *Ase*I restriction enzyme and the results showed that AA produced two fragments (25 and 187 bp), AT produced three fragments (25, 187 and 212 bp) and TT produced a single fragment (212 bp). Restriction products of each gene were separated on 3% agarose gels and visualized under ultraviolet light on a transilluminator.

Statistical analysis

In our study, the equation published by Dawson-Saunders and Trapp (1994) was used to calculate the sample size. Software, MedCalc ver. 11.6 and SPSS ver. 19.0 were used for statistical analyses. Allelic and genotypic proportions of *T-1131C APOA5* and *SG13S114* polymorphisms were calculated in cases and controls. Hardy–Weinberg equilibrium (HWE) was tested by chi-square (χ^2) or Fisher test. The odds ratio (OR) and 95% confidence intervals (CI) were used to assess the association of these polymorphisms with IS. Three different genetic models were applied: recessive, dominant and additive. Independent sample's *t*-test was used to evaluate the difference of average age. Binary logistic analysis comparing the two groups was used to assess the independent effect of polymorphisms and clinical characteristics of IS (subtypes, traditional risk and demographic factors). For all variables, P value less than 0.05 ($P < 0.05$) was considered statistically significant. To avoid false interpretation of P value (type 1 error) of genetic associations, Bonferroni correction has been performed (Dunn 1961).

Results

Genetic association study

Table 1 shows the genotype and allele frequencies of *T-1131C APOA5* and *ALOX5AP SG13S114* variants in IS groups and controls.

T-1131C APOA5: The distribution of *T-1131C APOA5* polymorphism was in HWE among cases ($X^2 = 5.44$, $P = 0.07$) and controls ($X^2 = 3.04$, $P = 0.22$). In light of our results, the genotypic frequencies of *T-1131C APOA5* gene

Table 1. Frequencies of *T-1131C APOA5* and *ALOX5AP SG13S114* polymorphisms in IS subjects and controls.

Genotype/allele	Case (%) ($n = 175$)	Control (%) ($n = 201$)	OR (95% CI)	*P*	*P* corrected
T-1131C APOA5					
TT	99 (56.6)	134 (66.7)	Ref.		
TC	57 (32.6)	58 (28.8)	1.33 (0.85–2.08)	0.212	
CC	19 (10.9)	9 (4.5)	2.86 (1.24–6.58)	0.013	0.039
TT + TC	156 (89.1)	192 (95.2)	Ref.		
CC	19 (10.9)	9 (4.5)	2.60 (1.14–5.90)	0.023	0.046*
TT	99 (56.6)	134 (66.2)	Ref.		
TC + CC	76 (43.4)	67 (33.3)	1.54 (1.53–2.33)	0.045	0.090
T	255 (72.9)	326 (81.1)	Ref.		
C	95 (27.1)	76 (18.9)	1.54 (1.01–2.33)	0.007	0.014*
SG13S114					
AA	89 (50.9)	119 (59.2)	Ref.		
AT	51 (29.1)	64 (31.8)	1.06 (0.67–1.68)	0.786	
TT	35 (20.0)	18 (9.0)	2.57 (1.49–4.83)	0.003	0.009*
AA + AT	140 (80.0)	183 (91.0)	Ref.		
TT	35 (20.0)	18 (9.0)	2.54 (1.38–4.68)	0.003	0.006*
AA	89 (50.9)	119 (59.2)	Ref.		
AT + TT	86 (49.1)	82 (40.8)	1.40 (0.93–2.11)	0.104	
A	229 (65.4)	302 (75.1)	Ref.		
T	121 (34.6)	100 (24.9)	1.59 (1.16–2.19)	0.004	0.008*

*Significant; Ref., reference; CC vs TT + CT, recessive model for *T-1131C APOA5*; TC + CC vs TT, dominant model for *T-1131C APOA5*; C vs T, additive model for *T-1131C APOA5*; TT vs AA + AT, recessive model for *SG13S114*; AT + TT vs AA, dominant model for *SG13S114*; T vs A, additive model for *SG13S114*.

in cases were 56.6% TT, 32.6% TC and 10.9% CC versus 66.7% TT, 28.8% TC and 4.5% CC for controls. T and C allele frequencies in cases and controls were 72.9, 27.1 and 81.1, 18.9%, respectively. After correcting the value of *P*, we noted that subjects harbouring the CC genotype were significantly associated with the risk of IS when compared to TT genotype (OR: 2.86; 95% CI = 1.24–6.58; Pc = 0.039). TC versus TT was not found to influence the risk of IS. When considering the genetic models, we noticed a significant association with IS risk in the additive model (C versus T, OR: 1.54; 95% CI = 1.01–2.33; Pc = 0.014) and in the recessive model (CC versus TT + TC, OR: 2.60, 95% CI = 1.14–5.90; Pc = 0.046), but the dominant model (TC + CC versus TT) showed no trend on the risk of IS. Thus, the presence of minor allele could be a genetic marker in the process of developing IS in Morocco.

ALOX5AP SG13S114: Distribution of *SG13S114* variant in controls ($X^2 = 4.35$, $P = 0.11$) did not deviate from HWE contrary to that observed in cases ($X^2 = 21.9$, $P < 0.01$). The distribution of genotypes among cases was 50.9% AA, 29.1% AT, 20.0% TT and 59.2% AA, 31.8% AT, 9.0% TT in controls. Allele frequencies in cases and controls were 65.4% A, 34.6% T and 75.1% A, 24.9% T, respectively. An increased risk of IS was observed with TT versus AA of *SG13S114* variant (OR: 2.57; 95% CI = 1.49–4.83; Pc = 0.009) but not with AT versus AA. Except for the dominant model (AT + TT versus AA), *SG13S114* variant was associated with an increased risk of IS both additive (T versus A, OR: 1.59; 95% CI = 1.16–2.19; Pc = 0.008) and recessive (TT versus AA+AT, OR: 2.54; 95% CI = 1.38–4.68; Pc = 0.006) models. Therefore, our results suggest that the T allele of *SG13S114* could influence the risk of IS. Thus, the size of the sample included in this work has enabled us to bring out an association of *T-1131C APOA5* and *ALOX5AP SG13S114* polymorphisms in IS risk with 80% power.

Characteristics of IS subjects

Table 2 represents the correlation between *T-1131C APOA5* and *ALOX5AP SG13S114* and IS subtypes. According to the TOAST classification, the different IS subtypes were 39.4% atherosclerosis, 26.9% cardioembolic, 6.3% lacunar and 27.4% for other causes. Genotype–phenotype correlation showed no positive trend.

Table 3 shows the distribution of *T-1131C APOA5* and *ALOX5AP SG13S114* polymorphisms as a function of demographic parameters and risk factors of IS patients. Male patients aged above 55 years were overrepresented when compared with female patients less than 55 years. There was no significant difference between *T-1131C APOA5*, *SG13S114* variant and demographic parameters such as age and sex. We have not found a positive association between cerebral IS factors such as hypertension, diabetes, smoking and alcohol consumption with the genotypes of *T-1131C APOA5*. In the contrast, a positive correlation was observed between hypertension and *SG13S114* variants of the *ALOX5AP* gene ($P = 0.012$).

Table 2. Distribution of *T-1131C APOA5* and *ALOX5AP SG13S114* variants according to IS subtype.

		T-1131C APOA5 (%)				*SG13S114* (%)			
	$n = 175$	TT	TC	CC	*P*	AA	AT	TT	*P*
IS subtype									
Atherosclerosis	69 (39.4)	39 (56.5)	22 (31.9)	8 (11.6)	0.725	33 (47.8)	17 (24.6)	19 (27.5)	0.270
Cardioembolic	47 (26.9)	27 (53.2)	17 (36.2)	5 (10.5)		23 (48.9)	17 (36.2)	7 (14.9)	
Lacunar	11 (6.3)	8 (72.7)	1 (9.1)	2 (18.2)		8 (72.7)	1 (9.1)	2 (18.2)	
Others	48 (27.4)	27 (56.3)	17 (35.4)	4 (8.3)		25 (52.1)	16 (33.3)	7 (14.6)	

Table 3. Distribution of *T-1131C APOA5* and *ALOX5AP SG13S114* according to risk factors of IS patients.

		T-1131C APOA5 (%)				*SG13S114* (%)			
Parameter	$n = 175$	TT	TC	CC	*P*	AA	AT	TT	*P*
Age									
$\leq$ 55 years	77	38 (49.4)	29 (37.7)	10 (13.0)	0.230	40 (51.9)	24 (31.2)	13 (16.9)	0.640
>55 years	98	61 (62.2)	28 (28.6)	9 (9.2)		49 (50.0)	27 (27.6)	22 (22.4)	
Gender									
Female	76	43 (56.6)	27 (35.5)	6 (7.9)	0.485	38 (50.0)	24 (31.6)	14 (18.4)	0.794
Male	99	56 (56.6)	30 (30.3)	13 (13.1)		51 (51.5)	27 (27.3)	21 (21.2)	
Hypertension									
Yes	88	50 (56.8)	28 (31.8)	10 (11.4)	0.963	49 (55.7)	17 (19.3)	22 (25.0)	0.012*
No	87	49 (56.3)	29 (33.3)	9 (10.3)		40 (46.0)	34 (39.1)	13 (14.9)	
Diabetes									
Yes	36	21 (58.3)	9 (25.0)	6 (16.7)	0.328	21 (58.3)	8 (22.2)	7 (19.4)	0.535
No	139	78 (56.1)	48 (34.5)	13 (9.4)		68 (48.9)	43 (30.9)	28 (20.1)	
Tobacco									
Yes	59	33 (55.9)	19 (32.2)	7 (11.9)	0.954	27 (45.8)	17 (28.8)	15 (25.4)	0.413
No	116	66 (56.9)	38 (32.8)	12 (10.3)		62 (53.4)	34 (29.3)	20 (17.2)	
Alcoholism									
Yes	14	6 (42.9)	5 (35.7)	3 (21.4)	0.347	9 (64.3)	3 (21.4)	2 (14.3)	0.577
No	161	93 (57.8)	52 (32.3)	16 (9.9)		80 (49.7)	48 (29.8)	33 (20.5)	

*Significant.

Discussion

This is the first study exploring the association of *T-1131C APOA5*, *ALOX5AP SG13S114* and IS risk in a sample of the Morocco population. Among the studied natural variants of *APOA5* gene, *T-1131C APOA5* polymorphism was widely believed to be involved in the process of development of cerebrovascular and cardiovascular disease (Szalai *et al.* 2004; Pi *et al.* 2012; Ouatou *et al.* 2014). In the light of our results, we also found that the C allele of *T-1131C APOA5* gene was associated with an increased risk of IS but the risk was 2.86-fold when the subject carried CC genotype. Similar findings were observed by Pi *et al.* (2012) who demonstrated in a meta-analysis that *T-1131C APOA5* polymorphism was associated with an increased risk of IS in European and Asian populations. Other previous studies have also found the same results (Maasz *et al.* 2008; Jaromi *et al.* 2010). According to the different genetic combinations, IS risk is increased in additive and recessive models but not in dominant model. This contrasts with the work of Pi *et al.* (2012) who have found a high risk in all genetic models. This discrepancy between the association of *T-1131C APOA5* with IS in genetic models may depend on the sample size and allelic frequencies between different populations worldwide. The mechanism by which this polymorphism could induce IS is that, it can affect the function of transcription of the protein which secondarily causes a change in the synergistic *APOA5* with lipoprotein lipase, the main enzyme involved in circulating triglycerides regulation (Pennacchio *et al.* 2001; Yang *et al.* 2004; Wright *et al.* 2006). In pathological conditions, the abnormal accumulation of lipids in endothelial cells could promote the establishment of atheromatous plaque that is elemental in the events leading to the development of IS (Hachinski *et al.* 1996; Hassan and Markus 2000; Havasi *et al.* 2006). In this study, we have found a negative correlation between *T-1131C APOA5* variant with demographic factors (age and gender). These results are consistent with those observed in Hungarian population (Jaromi *et al.* 2010) but discordant with the results obtained by Maasz *et al.* (2008) in the same

study population. These differences could be explained by the sample size and the criteria for selection of patients. With traditional risk factors such as hypertension, diabetes, smoking and alcoholism, we have not found an association consistent with *T-1131C APOA5*. These results do not match with those of Havasi *et al.* (2006) and Maasz *et al.* (2008). They showed that *T-1131C APOA5* allele was associated with IS risk factors. This discrepancy could be due to the fact that genetic risk factors may vary from one population to another. However, no positive correlation was observed in our study between *T-1131C APOA5* and IS subtypes. This contrasts with that observed in the literature (Havasi *et al.* 2006). Several factors could explain these contradictions among others such as, the size of the sample, different methods, distribution of IS subtypes in different populations and early diagnosis by computer tomography or MRI.

ALOX5AP gene plays a key role in the inflammatory process, in the production of leukotriene (Spanbroek *et al.* 2003). Among the haplotypes, *SG13S114*, the most frequently studied is involved in the pathogenesis of several cardio–cerebrovascular diseases (Gretarsdottir *et al.* 2003; Helgadottir *et al.* 2005; Gulcher *et al.* 2005; Kostulas *et al.* 2007). In the present study, we observed that TT genotype and the T allele of *SG13S114* variant were associated with an increased risk of IS. These results are consistent with the work done in some European populations such as Iceland, Germany and Spain (Helgadottir *et al.* 2004; Lõhmussaar *et al.* 2005; Domingues-Montanari *et al.* 2010). Further, Sharma *et al.* (2013) also found the same results in Indian population. However, a negative correlation of the *SG13S114* SNP with IS was observed in white American population (Meschia *et al.* 2005; Zee *et al.* 2006). In the present study, the recessive model of *SG13S114* has shown an association with a risk of 2.54-fold with IS, but the dominant model showed no trend. This was similar in part to the work of Wang *et al.* (2014) and Domingues-Montanari *et al.* (2010) who found that *SG13S114* was associated with an increased risk of IS in dominant as well as recessive models. In contrast, Zintzaras and Lau (2008) found no association in any genetic models. These discrepancies concerning the relation between SG13S114 variant and IS risk could be explained by the difference in sample sizes and genetic variability of ethnicities. This could also depend on the size of the study samples. In accordance with the classification of IS subtypes, no positive association was observed with the T allele of *SG13S114*. This finding was similar to what was reported in white American population (Meschia *et al.* 2005). In addition, it was reported in Chinese population that A allele of *SG13S114* variant was associated with an increased risk of atherothrombotic IS or small artery disease than other subtypes (Zhang *et al.* 2006; Ye *et al.* 2014). However, it has recently been revealed in the same Chinese population that there was a positive association of *SG23S114* with atherothrombotic IS forms (Wang *et al.* 2014). Although, *ALOX5AP* gene plays an important role in atherosclerosis and inflammatory diseases, but these inconsistencies by relationship *SG13S114* and IS subtypes may assume that atherosclerosis in stroke could be due to several mechanisms such as genetic (other variants of *ALOX5AP* gene) and pathophysiological (endothelial dysfunction, formation of atheromatous lesions and atherosclerotic complications) factors or synergy of two factors. In light of our results, according to demographic factors and risk factors for IS, there was no positive correlation with *SG23S114* with the exception of hypertension. These results were almost similar to those of Meschia *et al.* (2005), who found a significant association of *SG13S114* variants with hypertension, but not with other risk factors and demographic factors. The mechanism by which *ALOX5AP* gene may contribute to the occurrence of stroke is the activation of the inflammatory pathway mediated by leukotriene (Kostulas *et al.* 2007). This occurs with the changes in the local blood flow which causes release of mediators that are involved in vasodilatation, vascular permeability and leukocyte migration. Leukotrienes have also been implicated in the initiation and progression of atherosclerosis (Riccioni *et al.* 2010; Di Gennaro and Haeggstrom 2012).

In conclusion, the current study suggests that in Moroccan population, the *T-1131C APOA5* and *SG13S114* variants are independently associated with an increased risk of IS. Hypertension remains a major risk factor in the occurrence of stroke with *SG13S114*. The study of these polymorphisms in cerebral ischaemia is the first in Morocco, and it would be desirable in future studies to increase the sample size and evaluate these polymorphisms based on biochemical parameters.

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