Association between Ava II Polymorphism in LDL receptor gene and hyperlipidemicischemic stroke patients

ABSTRACT

Increased plasma level of low density lipoprotein-cholesterol (LDL-C) is a risk factor for atherosclerosis development and severity. The low-density lipoprotein receptor (*LDL-R*) is among one of the control mechanisms that the liver uses for cholesterol homeostasis. Gene variations in the *LDL-R* have been reported to cause hypercholesterolemia and consequently lead to cardiovascular diseases (CVD).The study investigates the association between polymorphism of the *LDL-R* gene and plasmalipid parameters levelsin patients with ischemic stroke.115 controls and 95 ischemic stroke patients matched for sex and age were enrolled in the study. Lipid profiles were measured using biochemical standards methods and lipoprotein ratios were calculated. AvaII (rs5925) genotypes were performed by PCR-RFLP combined with 2% gel electrophoresis.The frequencies of A^-A^-, A^-A^+ and A^+A^+ genotypes for*LDL-R* gene polymorphism were 73.04%, 23.48% and 3.48% for the control,18.95%, 72.63% and 8.42% for the ischemic stroke subjects (P<0.0001)respectively.The frequencies of A^- and A^+ alleles were 84.78% and 15.22% for thecontrol and 55.26% and 44.74% for the stroke subjects (P<0.0001) respectively.The study shows that the mutant allele of the *LDL-R* genewas associated withdyslipidemia for both subjects. The subjects with A^-A^- genotype had significant (P=0.0001-0.0063) lowerplasma levels of TC, TG, LDL-C, VLDL-C, Non-HDL-C, TC/HDL-C, TG/HDL-C, LDL-C/HDL-C, AIPand AC than the subjects with A^-A^+ and A^+A^+genotypes for both subjects.The effect of *LDL-R* gene Ava polymorphism on plasma lipid levels isassociationwith higher lipid parameters in both subjects.

KEYWORDS: LDL receptor gene; lipid parameters; ischemic stroke, PCR-RFLP.

INTRODUCTION

Acute ischemic stroke (AIS) is the fourth causes of death in the United States of America and is often characterized by sudden loss of blood flow to an area of the brain resulting in irreversible brain injury and leading to neurologic damage [1]. AIS is caused by embolic or thrombotic occlusion of a cerebral artery. Embolic AIS is caused as a result of the blockage of the cerebral artery by blood clots that is formed somewhere else in the body and travel to the brain. Whereas, Thrombotic AIS is caused by the blockage of the cerebral artery by blood clots that is formed within the brain and represents more than 50% of all acute ischemic stroke cases[1].Studies have shown that dyslipidemia is one of the major risk factors for stroke, and these include high levels of serum or plasma total cholesterol (TC) and triglycerides.(TG), low-density lipoprotein cholesterol (LDL-C), apolipoprotein B (ApoB), and low levels of high-density lipoprotein cholesterol (HDL-C) are high-risk factors for the development of acute ischemic stroke and the

progression of atherosclerosis [2,3].These plasma lipid parameters may be affected by environmental factors such as hypertension, diet, obesity, demographics, exercise, cigarette smoking, alcohol consumption and genetic factors [2,3].

Cholesterol is pooled by the liver from cholesterol that is synthesized in the cells or fromeaten diet. Increased serum or plasma levels of low density lipoprotein-cholesterol (LDL-C), is one of the most important risk factors for atherosclerosis development and progression and its lowering remains the primary target in the management of dyslipidemia[4]. The liver is the main organ that helps in cholesterol homeostasis and low-density lipoprotein receptor (*LDL-R*) is used as one of the regulating mechanisms [5]. LDL receptor plays an important role in cholesterol homeostasis as it regulates the uptake of LDL particles by the liver and delivers cholesterol to the adrenal glands and gonads for steroid hormone synthesis and to the liver for bile acid synthesis [6].*LDL-R* is a trans-membrane glycoprotein that plays an important role in the uptake of LDL-C from blood circulation in a process that is mediated by apolipoprotein B [7,8]. *LDL-R*binds at neutral pH specifically and with a high affinity to extracellular lipoprotein particles [9].The LDL–C and *LDL-R*complex is then brought into the cell by endocytosis [10].LDL–C is then released by the *LDL-R* at an acidic pH for degradation by a lysosome which results in the release of free cholesterol and the return of the *LDL-R* to the cell surface [9].The human LDL receptor gene is located on chromosome 19 and contains 18 exons separated by 17 introns [11]. More than 770 mutations in the *LDL-R* gene have been reported[12].Mutations in the *LDL-R* gene have been reported to cause familial hypercholesterolemia [13]. In the present study, I investigated the effect of Ava II (rs5925) polymorphism of the *LDL-R* gene on plasma lipid parameters in patients with ischemic stroke.

MATERIALS & METHODS

Study subjects

95 ischemic stroke patients' blood samples were collected for the study. All the patients had cerebral computerized tomography taken which showed cerebral infarction and they were confirmed by neurologists in LUTH to have ischemic stroke.The healthy control subjects consist of 115individuals with socio-economic status as the ischemic stroke patients. Blood samples were obtained in an EDTA and heparin vacutainer bottles from healthy individuals and stroke patients who have been fasting for 12 to 16 hours. All the ischemic stroke and control subjects

were given consent forms and questionnaire. Ethical approval was obtained from LUTH Research and Ethical Committee. Stroke and control subjects not willing to participate in the study were excluded from the study.

Lipid parameters and lipoprotein ratios

Blood was collected in lithium heparinize tubes and the plasma was separated from red blood cells by centrifugation at 1500 x g for 15 min at 4^{o}C. The Total Cholesterol (TC), Triglyceride (TG), and HDL-Cholesterol were assayed using Randox kits (RANDOX Laboratories Ltd., Ardmore, Diamond Road, Crumlin Co. Antrim, United Kingdom). LDL-C andVLDL-C were calculated by method described by Momoh et al[3].

VLDL-C =TG/5 and LDL-C=TC - HDL-C –VLDL-C

Non-HDL-Cholesterol = TC — HDL-C

Atherogenic ratios

Dyslipidemia was defined according to standard procedure described by Momoh et al [3].

Atherogenic ratios like:HDL-C/LDL-C, TG/HDL-C, Castellis risk index I (TC/HDL-C),Castellis risk index II (LDL-C/HDL-C), atherogenic coefficient [(TC - HDL-C)/HDL-C] and atherogenic index of plasma (log TG/HDL-C) were calculated usingmethod described by Momoh et al[3].

DNA analysis

Genomic DNA was isolated from blood leukocytes using DNA Qiagen kits according to manufacturer instructions. The extracted DNA was stored at 4°C until analysis. The quality and quantity of extracted DNA were determined using spectrophotometric method with NANODROP *1000*^{R} **(**Thermo Fisher Scientific, United States of America), which quantified the amount of extracted DNA in nanogram per microlitre (ng/µL) and assessed the quality (purity) based on the ratio of absorbance at 260nm:280nm for all the samples. The Genotyping of the *LDL-R* gene Ava □ polymorphismwas carried out usingpolymerase chain reaction and restriction fragment length polymorphism (PCR-RFLP) method described by Long et al[14].PCR amplification was carried out with forward primer 5'GTCATCTTCCTTGCTGCCTGTTTAG-3' and reverse primer 5'-GTTTCCACAAGGAGGTTTCAAGGTT-3'[14].Each amplification reaction was performed in a total volume of 25 µL, containing 3 µL of genomic DNA, 1.0 µL of each primer, 7.5 µL of ddH_2O, and 12.5 µL 2 × Taq PCR Master Mix (constituent: 0.1 U Taq polymerase/µL, 500 µM dNTP each and PCR buffer). The PCR Cycling conditions for LDL-R

gene SNPwas: initial denaturation at 94°C for 3 min, followed by 30 cycles of denaturation at 94°C for 30s, annealing at 55°C for 30s, and extension at 72°C for 60 s, with a final extension at 72°C for 5 min. After electrophoresis on a 2.0% agarose gel with 0.5 µg/mL ethidium bromide, the amplification products were visualized under ultraviolet light.The PCR aliquot (8 µL) was digested with 10 U of Ava enzymeat 37°C, followed by electrophoresis on 2% agarose gel stained with ethidium bromide, 0.5mg/ml in Tris-borate EDTA. After restriction enzyme digestion of the amplified DNA, the genotypes were identified by electrophoresis on 2.0% agarose gels and visualized with ethidium-bromide staining ultraviolet illumination.The genotyping of these samples were completely consistent.

Statistical Analysis

Data were presented as Mean ± SD; GraphPad prism computer software version 5.01 was used to compare lipid profiles and lipoprotein ratios levels between genotypes for control and stroke subjects respectively. Allele frequencies were determined via direct counting and the standard-goodness-of-fit test was used to test the Hardy-Weinberg equilibrium.One-way ANOVA *posthoc* Turkey´s test was used for comparing significantdifference between wild type and mutant genotypes for both separate subjects.A P-value < 0.05 was considered statistically significant.

RESULTS

Results of electrophoresis and genotyping

After the genomic DNA of the samples were amplified by PCR and imaged on 2.0% agarose gel electrophoresis, the PCR products of 228 bp were found in all the samples (Figure 1). The genotypes identifiedwere named according to the presence or absence of the enzyme restriction sites. The presence of the cutting site indicates the A^+allele; while its absence indicates the A^- allele (cannot be cut). A^+A^+genotypes were homozygotes for the presence of the site (141- and 87-bp). A^-A^+ genotypes were heterozygotes for the absence and presence of the site (228-, 141- and 87-bp), and A^-A^-genotypes were homozygotes for the absence of the site (228-bp)respectively (Figure 2 and 3). The genotypic distribution was consistent with the Hardy-Weinberg equilibrium.

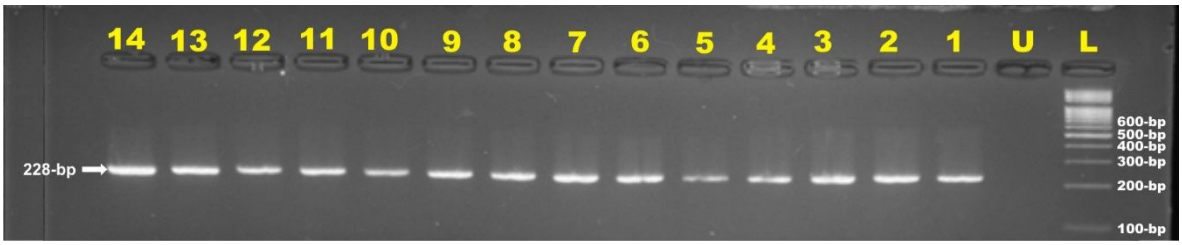

Figure 1. Agarose gel electropherogram for *LDL-R* gene PCR products of the DNA samples.Lane L: 100 bp marker ladder; lane U is non-template strand; lanes 1-14: the DNA samples of the 228 bp bands of the target gene.

Figure 2. Agarose gel electropherogram for genotyped *LDL-R* gene Ava □ polymorphism. Lane L, 100 bp marker ladder; lanes 16-24, 26, 28 and 29 are A^-A^- genotype (228-bp); lanes 25 and 27, A^-A^+ genotype (228-, 141- and 87-bp) and lanes 15 and 30 are non-template strands.

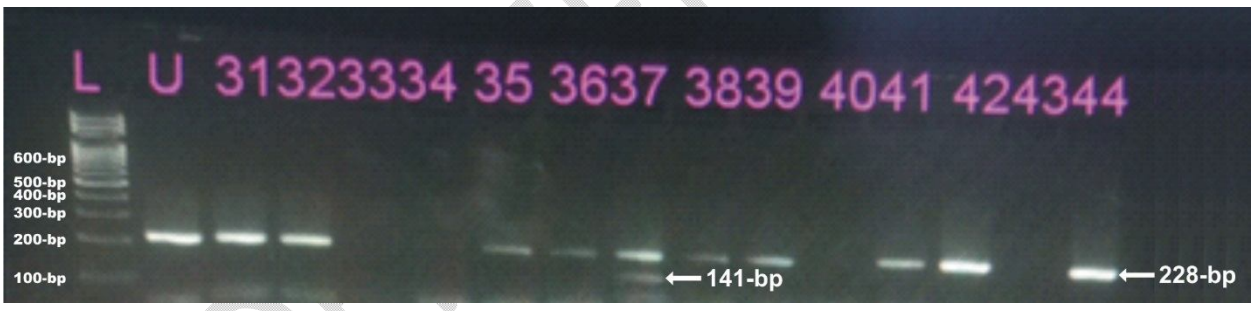

Figure 3. Agarose gel electropherogram for genotyped *LDL-R* gene Ava □ polymorphism. Lane L, 100 bp marker ladder; lanes U, 31,32, 35, 36, 38,39, 41, 42 and 44 are A^-A^- genotype (228-bp); lane 37, A^-A^+ genotype (228-, 141- and 87-bp) and lanes 33, 34, 40 and 43 are non-template strands.

Genotypic and allelic frequencies

The observed and expected genotypes of the examined *LDL* receptor gene polymorphism for the control and the stroke subjectswere examined according to Hardy-Weinberg equilibrium and no significant difference (P=0.8478 for control and P=0.5526 for stroke) were found between the observed frequencies and the expected Hardy-Weinberg frequencies for both subjects. The result

shows that the frequencies of A^- and A^+alleles were 84.78% and 15.22% for the control and 55.26% and 44.74% for the stroke subjects respectively (Table 1).

Table 1. . The number of observed and expected genotype of examined LDL-receptor gene for the control and stroke subjects according to Hardy-Weinberg equilibrium.

SNP	Genotype/ Allele Frequency	**Control subjects**		**Stroke subjects**	
LDL-R		Observed Frequency	Expected H-W Frequency	Observed Frequency	Expected H-W Frequency
	A^-A^-	84 (73.04%)	82.6630	18 (18.95%)	29.0132
	A^-A^+	27 (23.48%)	29.6739	69 (72.63%)	46.9737
	A^+A^+	4 (3.48%)	2.6630	8 (8.42%)	19.0132
P Value		0.8478		0.5526	
X^2 Value		0.9338		20.8881	
	A^-	195 (84.78%)		105 (55.26%)	
	A^+	35 (15.22%)		85 (44.74%)	

Plasma lipid profiles and lipoprotein ratios for both control and stroke subjects with different genotypes

The HDL-Cholesterol and HDL-C/LDL-Cholesterol were significantly (P<0.0001) higher in the A^-A^- genotype when compared to the mutants genotypes (A^-A^+ and A^+A^+) and other parameters were significantly (P<0.0001) lower in the A^-A^- genotype for the control subjects. For the stroke subjects, the mutant alleles (A^-A^+ and A^+A^+) have significant (P=0.0001 to 0.0063) higher TC, TG, VLDL-C, LDL-C, Non-HDL-C, TC/HDL-C, TG/HDL-C, LDL-C/HDL-C, AIP and AC values compared to the wild type (A^-A^-) genotype. No significant (P= 0.1993) difference was observed for the HDL-C/LDL-C values between the wild type and mutant alleles for the stroke subjects.

Table 2: The effect of LDL-Rgene genotypes on plasma lipid parameters for both control and ischemic stroke subjects

Parameters	Control subjects			Ischemic stroke subjects		
	Genotype			Genotype		
	A^-A^-(84)	A^-A^+(27)	A^+A^+(4)	A^-A^-(18)	A^-A^+(69)	A^+A^+(8)
TC (mg/dl)	158.20 ±6.25^c	161.80±7.09^b	191.90 ±2.05^a	203.72 ±6.16^c	228.11±6.87^a	219.63 ±5.08^b
TG (mg/dl)	91.35 ±6.436^c	117.01 ±7.39^b	127.40 ±5.40^a	163.71 ±5.43^b	192.22 ±7.14^a	189.32 ±6.83^a
HDL-C (mg/dl)	119.60 ±5.13^a	107.10 ±6.45^b	91.70 ±4.25^c	54.83 ±2.02^{ab}	56.45±2.78^a	52.92±1.82^b
VLDL-C (mg/dl)	18.27±0.39^c	23.40±0.47^b	25.48 ±0.32^a	32.74 ±1.37^b	38.44±1.44^a	37.86 ±1.41^a
LDL-C (mg/dl)	20.33±-1.54^c	31.30±-1.41^b	74.72±-2.36^a	121.15±3.93^c	133.22±4.68^a	128.85±3.62^b
Non-HDL-C(mg/dl)	38.60 ±2.02^c	54.70 ±3.24^b	100.20 ±-3.20^a	148.89 ±4.84^c	171.66 ±5.19^a	166.71 ±4.76^b

TC/HDL-C	1.323 ±0.087[c]	1.511 ±0.059[b]	2.093 ±0.042[a]	3.716 ±0.450[b]	4.041 ±0.396[a]	4.150 ±0.339[a]
TG /HDL-C	0.764 ±0.077[c]	1.093 ±0.084[b]	1.389 ±0.072[a]	2.986 ±0.197[b]	3.405 ±0.199[a]	3.577 ±0.213[a]
LDL-C/HDL-C	0.170 ±0.084[c]	0.292 ±0.072[b]	0.815±0.102[a]	2.210 ±0.135[b]	2.360±0.104[a]	2.435±0.098[a]
HDL-C /LDL-C	5.883 ±0.915[a]	3.422±0.611[b]	1.227 ±0.584[c]	0.453±0.061[a]	0.424 ±0.069[a]	0.411 ±0.058[a]
AIP	-0.117±0.007[c]	0.039±0.008[b]	0.143±0.010[a]	0.475 ±0.027[b]	0.532 ±0.028[a]	0.554 ±0.026[a]
AC	0.323±0.084[c]	0.511±0.097[b]	1.093±0.093[a]	2.715±0.217[b]	3.041±0.332[a]	3.150±0.316[a]

Data are presented as Mean ± SD (n=115 for control and 95 for stroke). TC, total cholesterol; TG, triglycerides; HDL-C, high-density lipoprotein-cholesterol; LDL-C, low-density lipoprotein-cholesterol; VLDL-C, very low-density lipoprotein-cholesterol; Non-HDL-C, Non-high-density lipoprotein cholesterol, AC, atherogenic index; TC/HDL-C, total cholesterol/high-density lipoprotein-cholesterol; LDL-C/HDL-C, low-density lipoprotein-cholesterol/high-density lipoprotein-cholesterol; TG/HDL-C, triglyceride/high-density lipoprotein-cholesterol,HDL-C/LDL-C, high-density lipoprotein-cholesterol;/low-density lipoprotein-cholesteroland AIP, atherogenic index of plasma.One-way ANOVA Posthoc Turkey´s test was used for comparing significantdifference between wild type and mutant genotypes for both separate subjects. a=highest, b= medium, c=lowest. Those genotypes that have the same letters are not statistically significant (P>0.05) while those that have different letters are statistically significant (P<0.05).

DISCUSSION

In this study, the polymorphism in the LDL receptor gene was determined by PCR-RFLP. The polymorphism in this study was presented in 81.05% (A^-A^+ =72.63% and A^+A^+ =8.42%) of patients with ischemic stroke with high lipid profile and 26.96% (A^-A^+=23.48% and A^+A^+=3.48%)of the non-symptomatic normolipidemic control subjects.TheA^+allele was significantly($P<0.05$) abundant in the stroke subjects compared with the control (44.74% vs. 15.22%) subjects, while the A^-allele was significantly ($P<0.05$) higher in the controlsubjects compared with the stroke (84.78% vs.55.26%). Different studies that are in-line with this study have showed that the frequency of A^+ allele was higher in patients with gallstone disease (Yu et al., 2002), phlegm-dampness constitution [15], acute myocardial infarction [16], and lower in patients with atherosclerotic cerebral infarction [17] when compared with healthy control subjects respectively. Salazar et al [18] found that the frequency of A^+ allele was significantly higher in 50 Familial hypercholesterolemia (FH) patients than in 130 healthy controls subjects (58% vs. 45%; P = 0.036). The FH patients had a higher frequency of A^+A^+ genotypes when compared to the normolipidemic controls (P = 0.0292) individuals. In another study carried out by Salazar et al[19] study shows that the frequency of A^+A^+ mutant genotype was higher in 170 white subjects presenting a lipid parameters with high risk for Coronary heart disease (CHD) compared to that of the 130 controls individuals (32% vs. 16%) from São Paulo City in Brazil.

For the control subjects, the homozygous wild type (A^-A^-) has significant ($P<0.001$) higher HDL-C and HDL-C/LDL-Cvalues compared with the heterozygous (A^-A^+) and homozygous (A^+A^+) mutant. The subjects with A^-A^- genotype also had significant (P=0.0001) lowerplasma levels of TC, TG, VLDL-C, LDL-C, Non-HDL-C, TC/HDL-C, TG/HDL-C, LDL-C/HDL-C, AIP and AC than subjects with A^-A^+ and A^+A^+ genotypes. The association of *LDL-R* gene Ava polymorphism and plasma LDL-C showed that there was a significant ($P<0.0001$) increase in LDL-C levels in A^+A^+ and A^-A^+ genotypes compared to A^-A^- genotype for the normolipidemic subjects. Pongrapeepornet al[20] research work showed that the mean LDL-C levels was slightly higher in the A^+A^+ genotype than the A^-A^+ and A^-A^- genotypes in 54 normolipidemic Thai subjects. Humphries et al[21] study showed that the individuals with one or two A^- alleles (absence of cutting site) had LDL-C levels that were lowered by 8.0% and 12.4%; respectively. Variation associated with this RFLP explained 4.2% of the sample variance.

The subjects with $A^-A^+$and A^+A^+genotypes, had significant (P=0.0001-0.0063) higherplasma levels of TG,TC, VLDL-C, TC/HDL-C, TG /HDL-C, LDL-C/HDL-C, AIP and AC than the subjects with A^-A^-genotype for the stroke subjects.The stroke patients' plasma levels of TC, LDL-C and Non-HDL-C significantly (P<0.0001) decreased from A^-A^+ to the A^+A^+ and finally to the A^-A^- genotypes. There was a significant (0.0005) increase in the plasma HDL-C level for the A^-A^+genotype compared to the A^+A^+ genotype while their HDL-C/LDL-C for the three genotypes did not show any significant (p=0.1993)difference.I observed an association between homozigosity and heterozigosity for the A^+ allele with greater dyslipidemia in patients with ischemic stroke. The results shows that A^+A^+ and A^-A^+ genotypes and A^+ allele are risk factor for ischemic stroke and A^-A^- genotype and A^- allele are protective factor against ischemic stroke.The difference in the association of *LDL-R* gene Ava polymorphism and plasmalipid parameters between the three genotypes may be as a result of the different *LDL-R* gene Ava polymorphism.Some studies did not show significant association between the *LDL-R* gene Ava polymorphism and serum or plasma lipid levels in Han children [22] or healthy controls[17,23].

CONCLUSION

The present study shows that there was significant difference in the genotypic and allelic frequencies between the normalipidemic and ischemic stroke subjects. The levels of LDL-C was significantly higher in the mutant alleles (A^-A^+ and A^+A^+) compared to the homozygous wild type (A^-A^-) for both subjects. The homozygous mutant has the highest LDL-C valueamong the three genotypes for both subjects.HDL-C was significantly higher in the wild type genotype compared to the mutant genotypes. Generally, higher lipid parameters were significantly higher in the mutant alleles ($A^+A^+$and A^-A^+) compared to the homozygous wild type (A^-A^-) for both the normalipidemic and ischemic stroke subjects respectively.

Limitation

This study has possible limitations due to its relatively small sample size, providing limited power to study holistic genetic effects and to detect interactions with other, potential relevant variables. Further studies are needed to definitely elucidate the impact of different genetic variability of the *LDL-R* gene in modulating lipid parameters and its association with stroke risk.

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