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Full Length Research Paper

Apo lipoprotein E4 gene APOE4: An early predictor of Dementia / Alzheimer's disease

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The present study aimed to determine the relationship of *APOE4* with socio demographic variables as well as with co-morbidities including hypertension and diabetes mellitus. In this study, 200 subjects with dementia were recruited, out of these 200 patients *APOE4* was present in 130 cases, 85 (65.4%) were male and 45 (34.6%) females. However, in 70 cases *APOE4* was missing, 41 (58.6%) males and 29 (41.4%) were females. Subjects who showed absence of *APOE4* were considered as control. Educational level and socioeconomic status were categorized as low, middle and high. BMI was considered as low or normal according to the standard criteria. 10 ml venous blood was obtained for genomic DNA extraction and genotyping for the Apo lipoprotein E (*APOE*) alleles using standard methods. A high percentage of patients with cognitive disorder showed the presence of *APOE4*. Among these patients males were more suffered than females. Quantitative characteristics showed that with increasing age the percentage of *APOE4* was increased, however, no effect on BMI was observed compared with the presence or absence of *APOE4*. A direct relationship of *APOE4* with diabetes was observed. In conclusion, presence of *APOE4* in male patients with cognitive disorder may increase the risk of dementia/Alzheimer's disease with middle socioeconomic status and education as well as diabetes. However no effect of BMI and blood pressure was observed.

Key words: Apo lipoprotein E4 Gene (*APOE4*), cognition, dementia, Alzheimer's disease.

INTRODUCTION

Neurological disorders are alarmingly rising worldwide; dementia is characterized by deterioration in cognition, function and behavior (Ganz, 2007). It may be latent due to brain damage or gradual due to some pathology. Alzheimer disease (AD) is the most ordinary cause of dementia (Jae-Min et al., 2010; Alzheimer's Association, 2010). In South Asia including India and Pakistan the prevalence of cognitive disorder is 1.9% which may be increased with age at seventh and eighth decades of life (Brookmeyer et al., 1998; Reitz et al., 2011). Cognitive areas of brain which are usually damaged in dementia/AD may comprise as reminiscence, speech, discernment skills, attention, productive aptitude, bearing

and problem solving aptitude (Ganz, 2007). Broadly, disease course of AD is classified as pre-dementia, mild or early cognitive impairment, moderate and functional impairments. Moreover, there is an advanced cognitive impairment and functional impairments (McCullaah et al., 2001).

Several risk factors are related to development of the disease, which include age, family history, head injury, gender, BMI, mild cognitive impairment, heart disease, hypertension, academic level, diabetes and presence of *APOE* etc (McCullaah et al., 2001). *APOE* is present in the chylomicron and Intermediate-density lipoprotein (IDLs) vital for the catabolism of triglyceride-rich

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lipoprotein ingredient. *APOE* is important in lipoprotein metabolism and cardiovascular disease. In addition it plays role in many biological courses which are linked to certain diseases like Alzheimer's disease, immunoregulation, and cognition (Singh et al., 2002).

APOE is produced mainly in the liver, but also found in tissues like brain, kidneys, and spleen (Mahley and Rall, 2000). In the nervous system, neurons show 6 to 7 receptors for *APOE* belonging to the low density lipoprotein receptor gene family (Zhang et al., 2010).

Apo lipoprotein E (*ApoE*) is a 34 kDa glycoprotein involved in lipid metabolism. The human *APOE* gene coding for this protein is polymorphic and is located on chromosome 19 (Das et al., 1985). There are three common co dominant alleles that encode three ApoE protein isoforms: E2, E3 and E4. These isoforms differ at the amino acid residues 112 and 158. Isoform E2 has cysteine residues at both sites, E4 has arginine residues at both sites, while E3, the most common form, has a cysteine at position 112 and an arginine at position 158 (Emi et al., 1988). Alzheimer's disease is mainly due to the aggregate of the peptide beta-amyloid. Apo lipoprotein E increases proteolytic activity of peptide beta amyloid, both inside and among cells (Jiang et al., 2008; Wildsmith et al., 2012). *APOE* is the known susceptible gene for late-onset of dementia and in sporadic form of AD. It accounts for as much as 20 to 30% of AD risk (Reitz et al., 2011).

Since there is not much study about dementia in Pakistan, there is lack of literature regarding the prevalence of dementia in Pakistan. However, there are observations which suggest that there could be many more cases of the dementia which go undiagnosed and unreported because it is thought that this is a natural consequence of the aging (Shafqat, 2008). The present study aimed to find out the relationship of *ApoE4* with socio demographic variables as well as with co-morbidities including hypertension and diabetes in the group of Pakistani people with cognitive impairment.

EXPERIMENTAL SECTION

200 subjects visiting the outpatient Department of Neurology of local hospitals of Karachi, Pakistan were included in the study. Ethical Committee of the Dow University of Health and Medical Sciences, Karachi, Pakistan approved the study. A written consent was obtained from all the participants. Exclusion criteria included non-genetic causes for dementias and other conditions that make the diagnosis of AD less likely or uncertain, such as focal neurological findings. All cases were diagnosed by a neurologist, who also reviewed all questionable cases in the study. Gender frequency-matched controls included those patients who showed the absence of *ApoE4*. Patient's Mini Mental Resources State Examination (MMSE) in the range of 24 to 27 was included in the study (Folstein et al., 1975). Educational level was categorized by years of schooling as low (Un-educated), middle (Secondary schooling), or high (Bachelor and Master Degree). The socioeconomic (SE) status was determined according to the monthly income and categorized as low, middle and high socioeconomic status. Subjects with duration of diabetes as >5.0

years were included in the study. Subjects were considered hypertensive if they have a blood pressure of 90/140 mmHg (Chobanian et al., 2003). BMI was calculated and considered as low or normal BMI according to the standard criteria (Kuczmarski and Flegal, 2000). Approximately 10 ml venous blood was obtained for genomic DNA extraction and genotyping for the Apo lipoprotein E (*APOE*) alleles by using standard methods (Wenham et al., 1991). PCR was performed using sequence-specific primers for the identification of *APOE* 2, 3 and 4 polymorphisms. For all PCR reactions (E2, E3, and E4), the presence of a 173-bp band indicated the presence of the specific *ApoE* haplotype. The control primer pair binds on chromosome 6 and therefore, a product of 785 bp was expected. A sample was considered negative for a particular *ApoE* haplotype when the haplotype-specific amplicon was absent and the 785-bp control amplicons was present. Absence of haplotype-specific and control amplicons in the same reaction was indicative of PCR amplification failure (Wenham et al., 1991). Data were recorded and analyzed with SPSS version 15.0 for Windows. The percentage count was calculated. Association of patient characteristics at base-line and presence or absence of *ApoE* was tested using chi-square and Student's t-test. $P \leq 0.05$ was taken as significant.

DNA extraction

To 0.5 ml of whole blood, 0.9 ml of 1X RBC lysing solution (0.32 M Sucrose, 1% Triton X-100, 5 mM $MgCl_2 \cdot 6H_2O$, 12 mM Tris- HCl, pH 7.6) was added and centrifuged at 13000 rpm for 1 min. The supernatant was discarded and the pellet was re-extracted with 0.9 ml RBC lysing solution. After centrifugation at 13,000 rpm, the pellet was washed with 1 ml water and allowed to dry. To the dried pellet, 20 μ l of 20% SDS, 80 μ l Proteinase K buffer (0.375 M NaCl, 0.12 M EDTA, pH 8.0) and 40 μ l of 10 mg/ml Proteinase K was added to the solution was incubated at 55°C for 1 h. Subsequently, 100 μ l of 6 M NaCl was added to the sample and centrifugation was carried out for 5 min at 13000 rpm. Supernatant was then transferred to a fresh tube and added with 1 ml 100% ethanol. DNA was then pelleted by centrifugation at 13000 rpm for 5 min. The DNA pellet was washed with 70% ethanol, air-dried, re-suspended in 50 μ l of 1X TE buffer, and stored at -20°C.

Allele-specific polymerase chain reaction

PCR was performed using sequence-specific primers for the identification of *APOE* 2, 3 and 4 polymorphisms. The sequence-specific forward and reverse primers were combined in three haplotype-detecting reaction mixtures "Primer Mix E2 (primers 1 and 3), E3 (primers 1 and 2), and E4 (primers 2 and 4 in Table 3). Because this genotyping system is based on the presence or absence of PCR amplification by allele-specific primers, it was imperative to ensure PCR amplification for those reactions that do not produce haplotype-specific amplicons. For this reason, each ApoE-specific Primer Mix also contained a pair of "control primers" (primers 8 and 9 in Table 3), which amplify a region of chromosome 6 in the HLA-DR locus, to verify PCR amplification in the absence of haplotype specific amplification in each PCR reaction.

For each DNA sample, 5 μ L of each Primer Mix (E2, E3, and E4) was placed in the bottom of three 0.2-mL PCR tubes. To this we added a mixture of 8 μ L of DNA (resuspended in DNase-free water or Tris buffer) and PCR reaction mixture containing PCR buffer, $MgCl_2$, deoxynucleotide triphosphate mixture, and *Taq* DNA polymerase. Thus, each 13 μ L PCR reaction contained the amount of the primers indicated in Table 1 and in addition, 1x PCR Buffer, 2 mM $MgCl_2$, 0.32 U of *Taq* DNA polymerase, 150 μ M each deoxynucleotide triphosphate, and 0.08 to 0.15 μ g of genomic DNA. Amplification was performed on an Eppendorf thermal cycler using

Table 1. Baseline quantitative characteristics stratified by presence and absence of *APOE4* allele.

Parameters	Subjects whom <i>ApoE4</i> was present (mean±SD)	Subjects whom <i>ApoE4</i> was absent (mean±SD)
Age (years)	71.45±7.10	66.10±6.56*
BMI (Kg/m ²)	24.34±3.33	24.79±2.67
SBP (mmHg)	143.08±17.49	142.93±18.65
DBP (mmHg)	88.22±7.44	86.81±9.04
Fasting Blood Sugar (mg/dl)	155.24±36.37	140.29±36.92*

SBP=Systolic blood pressure; DBP=diastolic blood pressure; *P-value <0.05 obtained using independent t- test considered as significant.

Table 2. Baseline qualitative characteristics stratified by presence and absence of *APOE4* allele.

Parameters		<i>APOE4</i> displayed (%) 130 (100)	<i>APOE4</i> not displayed (%) 70 (100)	P-value
Gender	Female	45 (34.6)	29 (41.42)	0.341
	Male	85 (65.4)	41 (58.57)	
Educational status	Masters	27 (20.76)	4 (5.71)	0.02*
	Bachelor	47 (36.15)	30 (42.85)	
	Secondary schooling	40 (30.76)	21 (30)	
	Uneducated	16 (12.30)	15 (21.42)	
Socio-economic status	Lower	24 (18.46)	15 (21.42)	0.52
	Middle	77 (59.23)	44 (62.85)	
	Upper	29 (22.30)	11 (15.71)	

*P-value <0.05 obtained using chi square test considered as significant.

a high-stringency touchdown-PCR protocol with high annealing temperatures to ensure specificity of amplification.

The PCR cycling condition were as follows: initial denaturation for 1 min at 96°C; followed by 5 cycles of 20 s at 96°C, 45 s at 70°C, and 25 s at 72°C; 21 cycles of 25 s at 96°C, 50 s at 65°C, and 30 s at 72°C; 4 cycles of 30 s at 96°C, 60 s at 55°C, and 120 s at 72°C. The PCR products were analyzed by electrophoresis on 1% Agarose gels and visualized under ultraviolet illumination.

For all PCR reactions (E2, E3, and E4), the presence of a 173-bp band indicated the presence of the specific *ApoE* haplotype. The control primer pair binds on chromosome 6 and therefore, a product of 785 bp was expected. A sample was considered negative for a particular *ApoE* haplotype when the haplotype- specific amplicon was absent and the 785-bp control amplicons was present. Absence of haplotype-specific and control amplicons in the same reaction was indicative of PCR amplification failure.

RESULTS AND DISCUSSION

Table 2 showed qualitative characteristics of patients with cognitive disorder. It was observed that out of 200 patients, *APOE4* was present in 130 patients. Among these 130 patients 85 (65.4%) male and 45 (34.6%) were females. 16 (12.3%) were uneducated, 40 (30.76%) were Secondary schooling, 47 (36.15%) were Bachelor and 27 (20.76%) subjects having Master's degree. According to

socioeconomic (SE) status, 24 (18.46%) subjects belong to lower SE status, 77 (59.23%) belong to middle SE status and 29 (22.3%) belong to upper SE status.

Absence of *ApoE4* was also observed in patients with cognitive disorder. It was observed that 70 patients showed the absence of *ApoE4*. Among these 29 (41.42%) were females, 41 (58.57%) were males. 15 (21.42%) were uneducated, 21 (30%) were Secondary school level, 30 (42.85%) were Bachelor and 04 (5.71%) subjects having Master's degree. According to socioeconomic (SE) status, 15 (21.42%) subjects belong to lower SE status, 44 (62.85%) belong to middle SE status and 11 (15.71%) belong to upper SE status.

Comparison of qualitative characteristics with presence and absences of *ApoE4* showed that although the count of socio-demographic including gender (male/females), education and socioeconomic status were more in the patients who showed the presences of *APOE4* as compared to the patients who showed the absence of *ApoE4*, significant difference (P<0.05) using chi square test was only observed in case of patients having Bachelor degree. Table 1 showed the quantitative characteristics of patients with cognitive disorders. It was observed that the mean age of patients with *ApoE4* was

Table 3. Primers required for *APOE* haplotype detection by SSP-PCR analysis.

Primer	Primer sequence	Primer identifies	Orientation	Final amount in each 13 ul PCR reaction (ng)
MDL Primer 8	TGC CAA GTG GAG CAC CCA A	Control HLA DBR1	Forward	6.5
MDL Primer 9	GCA TCT TGC TCT GTG CAG AT	Control HLA DBR1	Reverse	6.5
MDL Primer 1	CGG ACA TGG AGG ACG TGT	APOE-112 CYS	Forward	50
MDL Primer 2	CTG GTA CAC TGC CAG GCG	APOE-158 ARG	Reverse	50
MDL Primer 3	CTG GTA CAC TGC CAG GCA	APOE-158 CYC	Reverse	50
MDL Primer 4	CGG ACA TGG AGG ACG TGC	APOE-112 ARG	Forward	50

71.45±7.10 years. Their mean BMI was 24.34±3.33 kg/m² with systolic blood pressure (SBP) 143.0±17.49 mmHg and diastolic blood pressure (DBP) 88.22±7.44 mmHg.

Blood sugar level of these patients was 155.22±36.37 mg/dl. On the other hand, mean age of patients without *ApoE4* was 66.10±6.56 years. Their mean BMI was 24.79±2.67 kg/m² with systolic blood pressure (SBP) 142.93±18.65 mmHg and diastolic blood pressure (DBP) 86.81±9.04 mmHg. Blood sugar level of these patients was 140.29±36.92 mg/dl.

Comparison of quantitative characteristics with presence and absences of *ApoE4* showed that although the mean±SD of age, BMI, blood pressure (both SBP and DBP), level of blood sugar was more in patients who showed the presences of *ApoE4* as compared to patients showed the absence of *ApoE4*, significant difference ($P<0.05$) using student 't' test was only observed in case of age and blood sugar level of patients.

Figure 1 is a gel electrophoresis diagram and a representative of present study. In initial well, the ladder marker was loaded with a range from 50 to 785 base pairs (bp). Patient's samples were loaded from well 1 to well 10. It was observed that sample loaded in wells 1, 2, 3, 5, 6, 7 and 8 showed the positive *APOE4* polymorphism with 173 bp products. While sample loaded in well 4, 9 and 10 showed negative *APOE4* polymorphism.

Dementia refers to a disease state, which shows a decline in cognitive changes with advanced age, especially the memory. Apo lipoprotein E plays a specific role in the central nervous system, including neuronal development, regeneration and certain neurodegenerative processes (Brecht, 2004). Quantitative characteristics of patients showed that high percentages of patients with cognitive disorder showed the presence of *APOE4*. Among these patients males suffered more than females. A study suggests that patterns of cognitive decline and incidence of AD are similar in older men and women (Barnes et al., 2003). However, in another study the ε4 allele accounted for one third of Alzheimer's disease cases among men, but only one tenth among women (Qiu et al., 2004).

Explanations for the educational effect include increased brain reserve (Katzman, 1993), confounding by

indicators of socioeconomic status linked to education, such as diet, lifestyle, and occupational history (Mortimer and Graves, 1993). Educational status showed that patients with Bachelor degree and Secondary schooling were more sufferers than uneducated and highly educated. It was observed that a small percentage of uneducated patients were sufferers. Reason may be that these uneducated patients may be less sensitive or careless about their status. It is also observed that *APOE4* was present in small number of patients with low socioeconomic status while high percentage of patients was observed having middle SE status. The present study is in contrast with number of studies in which *APOE* epsilon4 carriers show decreased MMSE scores in uneducated people as compared with well-educated epsilon4 non-carriers (O'Bryant et al., 2008).

Quantitative characteristics showed that with increasing age, the percentage of *APOE4* was increased. Our study is in accordance with a number other studies who studied subjects between the ages of 75 to 85 years. A study stated that the presence of an *APOE4* allele is associated with impaired cognitive function, clinical dementia, AD, and mortality in about 5 years (Tilvis et al., 1998). Our study is in line with that of Raber et al. (2004) who observed that *ApoE4* allele may account for 95% of AD. Furthermore, scientists estimate that *APOE*-ε4 may be a contributing factor in the pathogenesis of the Alzheimer's disease (Ghebranious et al., 2011). Another study reported that carrying at least one *APOE4* allele was a risk factor for the development of mild cognitive impairment, but that once impairment was established; the gene has no effect on the risk of progressing to Alzheimer's disease (Barabash et al., 2009). Additionally it is stated that though the *APOE* epsilon 4 allele is a risk factor for Alzheimer's disease, there is no proof of a strong association between *APOE* epsilon 4 dosages and rate of cognitive decline (Murphy et al., 1997).

However no effect on BMI was observed when compared among patients with presence and absence of *ApoE4*. Our study is in contrast with Gorospe and Dave (2007) study which observed that BMI may be the vascular risk factors that might play a role in the development of Alzheimer's disease and dementia. We observed that patients with *ApoE4* have a significantly high



Figure 1. Genetic polymorphism *APOE4* in patients. Key: Ladder- 50 bp ladder marker; Lanes: 1, 2, 3, 10, 15, 17, 19 (No Polymorphism); Lanes: 4, 5, 6, 7, 8, 9, 11, 12, 13, 14, 16, 18, 20 (*APOE4* Polymorphism).

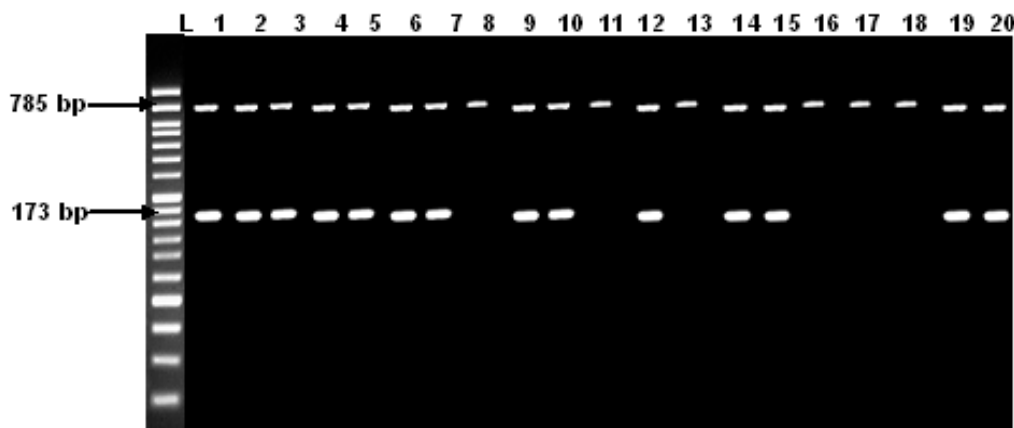


Figure 2. Genetic polymorphism *APOE4* in patients. Key: Ladder- 50 bp ladder marker; Lanes: 8, 11, 13, 16, 17, 18 (No Polymorphism); Lanes: 1, 2, 3, 4, 5, 6, 7, 9, 10, 12, 14, 15, 19, 20 (*APOE4* Polymorphism).

($P < 0.05$) blood sugar level than patients without *ApoE4*. Velayudhan et al. (2010) observed that the people who developed dementia were older, had a greater incidence of diabetes and an extended duration of diabetes. Both cognitive impairment and diabetes may be important self-determining risk for the cognitive impairment to dementia. The most important common mechanism between insulin-resistant (type II) diabetes and AD could be impaired insulin signaling; a form of toxic amyloid can damage neuronal insulin receptors and affect insulin signaling and cell survival (Sun and Alkon, 2006).

We observed a direct relationship of *ApoE4* with blood pressure. Our study is in line with that of Bender Andrew and Raz (2012) who observed that elevated pulse pressure was associated with poorer memory but only in the carriers of *APOE4*. Another study reported that *ApoE4* allele was considerably associated with

hypertension. This effect was found more in Asians (Niu et al., 2009). It is known that hypertension is a direct risk factor for vascular dementia and studies have suggested hypertension also impacts upon the prevalence of Alzheimer's disease. However there is no proof that lowering blood pressure prevents the development of dementia or cognitive impairment in hypertensive patients (McGuinness et al., 2009). Fuzikawa et al. (2008) also provide evidence that the *ApoE* genotype is not associated with prevalent hypertension in old age.

Conclusion

This study revealed presence of *APOE4* especially in male patients with cognitive disorder may increase the risk of dementia/Alzheimer's disease with middle socioeconomic

status and education as well as diabetes. However no effect of BMI and blood pressure was observed.

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