Annals of R.S.C.B., ISSN:1583-6258, Vol. 25, Issue 6, 2021, Pages. 5694 - 5705
Received 25 April 2021; Accepted 08 May 2021.

Chromosomal Alterations and Genotyping of Apo E4 in Patients with Alzheimer's Disease and Cardiovascular Disease a Particular Association

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ABSTRACT

Alzheimer's disease (AD) has as its core attribution in the decline of cerebral functions. Though, its etiology complex a perceptive is made for understanding its neurological, infectious, biochemical, genetic and cytogenetic mechanisms. Considering this, the study aimed to investigate the presence of chromosomal alterations in the peripheral blood lymphocytes and the genetic polymorphism of *APO E* was investigated using the PCR-RFLP technique also to verify if there was a high frequency of these alterations in patients diagnosed with AD, cardiovascular disease (CVD) and control. A total of 60 subjects, corresponding to 20 AD, 20 CVD and 20 controls were recruited for this study from Hospitals in Coimbatore. The mean ± SD values of chromatid type aberrations (CTAs) and chromosomal type aberrations (CSAs) for AD, CVD and control subjects were studied CTAs, CSAs and CA level of CVD subjects shows higher when compared to AD and control subjects. Therefore, the karyotypes of patients with AD, CVD undergo chromosomal alterations at different levels and they emphasize the importance in these cytogenetic investigations the routine management along with the association between CVD risk and AD. Carriers harbouring *APO E E4/E4* mutations are at higher risk of disease and those with the *APO E E3/E4* lowest risk, who represent the bulk of families with Alzheimer's disease, typically harbour gene mutation.

KEYWORDS: Alzheimer's disease, Cardiovascular Disease, Chromosomal aberration and *APO E* polymorphism.

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INTRODUCTION

One of the most widespread ranges of dementia in elderly adults is Alzheimer's disease (AD). The foremost features of this disease is the worsening of cerebral functions, with the episode of memory loss, which can irreversibly progress, affecting the motor functions, even leading to the death of the individual [1]. The neuropathological changes are mainly the formation of neurofibrillary tangles and altered amyloid metabolism, which causes synaptic loss and neuronal death [2]. The etiology of AD is still considered complex, despite the progress made in the understanding of its biochemical and genetic mechanisms [3]. Genetic studies have highlighted four genes responsible for AD: *Amyloid precursor protein (APP), Presenilin-1*, *Presenilin-2 (PSEN-1* and *2) and Apolipoprotein E (APO E)* [4]. Although the discovery of these genes raised enthusiasm for identifying the genetic basis of late-onset Alzheimer's disease (LOAD), no causative variants have been reported to date. The *APOE E4* allele (19q13.3 region) remains the main single genetic risk factor for AD development [5]. *APO E E4* causes the sporadic cause of AD and which accounts for more than 99% of AD cases, and it has a gene-dose effect on the risk and age of disease onset [6, 7]. The intriguing facet between cardiovascular risk factors and AD association is the occurrence. During ageing, the prevalence of dementia, especially AD and CVD, increases and its precursor clinical stage: mild cognitive impairment [8, 9, 10].

This study aimed to perform cytogenetic analysis by conventional staining and G-banding was used to investigate the presence of chromosomal alterations in peripheral blood lymphocytes and the genetic polymorphism of the *APO E* was investigated using the PCR-RFLP technique and also to verify whether there was a high frequency of these alterations in AD, CVD and control patients. The present study has been designed to find the basic genetic characteristics of AD, CVD and control patients to enable the different disease risk.

MATERIALS AND METHODS

Subject Recruitment and Sample Collection

Informed consent was obtained from all the participants. Institutional Ethical Clearance was obtained from the Institutional Ethical Clearance Committee, Bharathiar University, Coimbatore, Tamil Nadu, India. A total of 60 subjects, corresponding to 20 AD, 20 CVD and 20 controls were recruited for this study from Hospitals in and around Coimbatore. About 5ml of the blood samples were collected from the test subjects and the healthy ageing adult's subjects after 12 hours of fasting. Samples were collected in a heparin

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tube and were brought to the culture laboratory in sterile and tightly covered ice packed plastic containers for carrying out chromosomal, genotypic and biochemical analysis.

Cytogenetic Analysis

Cultures of leucocytes were obtained from the peripheral blood following method [11]. 0.5ml blood was added to 4.5ml RPMI 1640 medium supplemented with ten per cent fetal bovine serum, 2mM l-glutamine, one per cent streptomycin-penicillin, 0.2ml reagent grade phytohemagglutinin, and was incubated at 37ºC for 72nd hours. At the 71st hour, cultures were treated with 0.1g/ml colcemid to block the cells in the metaphase stage. Lymphocytes were harvested after the 72nd hr by centrifuging the cells to remove the culture medium (800-1000 rpm). Hypotonic solution (KCl 0.075 M) was added and incubated at 37ºC for 20 minutes to swell the cells, then the cells were treated twice with fixative (3:1 ratio of methanol : acetic acid). The lymphocytes were then added onto the slides carefully and dried on a hot plate (56ºC, 2 min). Finally, the slides were stained using the Trypsin-Giemsa technique. For the CA analysis, 50 metaphases in the first cell cycle were evaluated per subject under a microscope (100X) to identify the numerical and structural chromosomal abnormalities. Observations were recorded.

Genotyping

Whole genomic DNA was collected by following the kit protocol (Bangalore Genei-blood DNA extraction kit). The whole blood was collected in EDTA tubes to avoid the coagulation of blood. The first step in the extraction procedure was the lysis of the RBC using solution. In molecular biology, agarose gel electrophoresis routine and a vital part which separates DNA fragments based on their molecular weight the digested fragments were separated by electrophoresis using one per cent agarose at 150V for few hours. After electrophoresis, the polymorphic patterns were analyzed. Standard DNA was prepared at various concentrations (10, 25, 50 and 75µg/mL). The control DNA was serially diluted in distilled water. 50µL of the isolated genomic DNA was diluted in 1mL distilled water and OD was measured at 260nm using a spectrophotometer.

APO E gene polymorphism

Each PCR reaction mixture contained (~200 ng) of the prepared DNA template, 1× PCR buffer, 3.0mM MgCl2, 2.0mM of each of the primers, and 200mM of each deoxynucleotide triphosphate (dNTP); 1.25 U was the final concentration of Taq DNA polymerase (Himedia) and DMSO (5%) in a thin-walled microfuge tube. The PCR products were electrophoresis on one per cent agarose gels containing EtBr and viewed under ultraviolet light.

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Forward Primer **TCCAAGGAGCTGCAGGCGGCGCA**

Reverse Primer **GCCCCGGCCTGGTACACTGCCA**

Restriction digestion

By using restriction enzyme the allelic variants were identified that differentiate between alleles. For digestion of PCR product with Afl III (5.000 U/ml) and Hae II (20.000 U/ml), 10xbuffer, and 0.2 µl BSA the following protocol was used directly after amplification: PCR reaction product 10µL, Nuclease free water 18µL, 10X buffer R 2µL and Afl III enzyme 2µL and Hae II enzyme 2µL. The contents were incubated for 24 hours at 37°C.

Statistical Analysis

Statistical analysis was performed with SPSS V 22.0 (IBM corp., Armonk, N.Y., USA) statistical software package. The data was entered on a spreadsheet and checked for error and stored. The group statistics for subjects such as Mean ±SD were assessed.

RESULTS

In this study of 60 subjects, corresponding to 20 AD, 20 CVD and 20 controls were analyzed. The detailed chromosomal aberration (CA) for the individual samples was analyzed. **Table 1** depicts the comparative analysis of CA for the AD patients, CVD patients and the control with and the mean ± SD values of the age groups. The mean±SD values of chromatid type aberrations (CTAs) and chromosomal type aberrations (CSAs) for AD, CVD and control subjects were (65.00±3.17, 61.00±4.59, 63.00±4.25); CTAs (3.00±1.48, 4.15±1.81, 0.95±1.05); CSAs (1.30±0.73, 2.00±1.12, 0.55±0.51) and CA (4.15±1.92, 6.15±2.85, 1.50±1.27). The CTAs, CSAs and CA level of CVD subjects show higher when compared to AD and control subjects. Linkage analysis and genome-wide association studies have provided fundamental insights into the development of coronary artery disease and myocardial infarction. The detailed molecular analysis will lead to understanding how the disease-causing genes tend to increase the risk of CVD.

Totally 40/60 patients were seen with chromosomal anomalies. The aberrations were: Chromatid type aberrations in AD (21q) (21q, 14q) (21q, 14q, 19q) and (1q, 14q, 19q) In CVD subjects 9q deletion and ring formation in 13^{th} chromosome along with chromatid aberrations in 2q dicentric and 3q break. The present study of CVD patients has revealed an increased chromosomal instability on specific chromosomes in lymphocytes. The most commonly seen genetic alterations were found in chromosome numbers 9 & 21. The other

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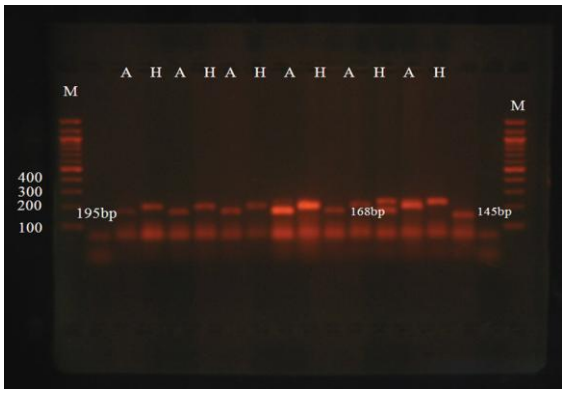
chromosomes such as 2, 3q, 4p, 12, and 13 showed minor alterations. Since it is an inherited disorder until yet there is no proper cure for this disease

Table 1: Comparative analyses of chromosome aberrations in Alzheimer's disease, Cardiovascular disease patients and control subjects (Mean ± S.D)

S. No	Particulars	Alzheimer's disease	Cardiovascular disease	Control
1	Age	65.00±3.17	61.00±4.59	63.00±4.25
2	Chromatidtype aberration	3.00±1.48	4.15±1.81	0.95±1.05
3	Chromosomal type aberration	1.30±0.73	2.00±1.12	0.55±0.51
4	Total	4.15±1.92	6.15±2.85	1.50±1.27

The PCR product which has been obtained from the *APO E* gene generally yields a 218-bp-long double-stranded DNA fragment. For each reaction, a negative control sample was used along with water instead of template DNA. To estimate the size of the amplicon by comparing it with a DNA marker (100bp ladder) this was run on the same gel. A representative photograph of the *APO E* PCR amplification product is illustrated in **Figure 1**. Lane 1 in the figure shows the 100 bp ladder, lane 3 contains a negative control, and the other lanes show the 218 bp *APO E* amplicon.

Figure 1 *Afl III* (A) and *Hae II* (H) digestion of the *APO E* 218 bp amplified fragment. M - 100 bp size marker. *E3* = 145 bp, *E2* = 168 bp, *E4* = 195 bp



In **Table 2**, the *APO E* genotype frequencies in the control subjects were 30% for the *E2* allele, 60% for the *E3* allele, and 10% for the *E4* allele. The frequencies in the CVD group were 20% for the *E2* allele, 20% for the *E3* allele, and 60% for the *E4* allele. The frequencies in the AD group were 10% for the *E2* allele, 30% for the *E3* allele, and 60% for the *E4* allele.

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In **Table 3** the distribution of the subjects according to *APO E* genotypes was as follows: control subjects were 20% for the *E2/E3* allele, 70% for the *E3/E3* allele, and 10% for the *E3/E4* allele. The frequencies in the CVD group were 20% for the *E2/E3* allele, 20% for the *E3/E3* allele, 40% for the *E3/E4* allele and 40% *E4/E4* allele. The frequencies in the AD group were 50% for the *E3/E3* allele, 10% for the *E3/E4* allele and 40% *E4/E4* allele The allele frequencies *E4* were also significantly different between the AD, CVD and control patients.

Table 2 Frequency of *APO E* alleles among the Alzheimer's disease, Cardiovascular disease patients and control subjects

Alleles	Alzheimer's disease	Cardiovascular disease	Control
	Percentage	Percentage	Percentage
E2	10 %	20 %	30%
E3	30 %	20 %	60 %
E4	60 %	60 %	10%
Total	100%	100%	100%

Table 3 Distribution of the APO E genotypes among the Alzheimer's disease, Cardiovascular disease patients and control subjects

Alleles	Alzheimer's disease	Cardiovascular disease	Control
	Percentage	Percentage	Percentage
E2/E3	-	20%	20 %
E3/E3	10 %	20%	70 %
E3/E4	50 %	40%	10 %
E4/E4	40%	40%	-
Total	100%	100%	100%

DISCUSSION

AD a chronic neurodegenerative disorder, which affects the cognitive functions and the individual's behaviour, social and professional life [12]. Chromosome studies in AD subjects revealed the changes in the chromosome and these changes can be noted in karyotypes of AD subjects further suggesting that these changes are associated with the disease [13, 14].

Chromosome defects are recognized as being the basis of several human genetic diseases [15]. AD accounts for more than seventy per cent of dementia cases hence it is necessary to recognize the symptoms in advance before it gets worse. Researches on

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cytogenetics are getting lesser and the screening of polymorphisms has been given importance in AD. Cytogenetic analysis on the AD patients has been carried out in many laboratories which reported evidence for linkage at 9p21.3, 9q22.33, 10q21.2 and 19q13.32 chromosomes [16]. Genetic damage is observed as variation in either the change in the number of chromosome and structure. Alterations in chromosome structure may be observed cytogenetically as chromosome aberrations. Multiple genome scans have found evidence for linkage to chromosomes 9, 10 and 19 [17, 18]. One of the core risk factors for AD neuropathogenesis is the instability of chromosome which results in either the addition or deletion of chromosome in a tissue thereby causing adverse effects in the physiology of an organism [19].

Cytogenetic analyses performed in the present study with patients diagnosed with AD and CVD revealed chromosomal type alterations and Chromatid type aberrations. On the other hand, the presence of both type aberrations was observed in the karyotypes of AD and CVD patients diagnosed with AD. When comparing these results with the healthy elderly, no type of chromosomal alteration and chromatid type aberration was observed, presenting with only normal metaphase cells (46 XX and 46, XY). Interestingly, in our study observed mutations in the chromosomes 10q, 9p, 9q, 14p 19q and 21, also some chromatid type aberrations have been observed has reported in the previous studies by [20,21] One of the most frequent abnormalities reported in cytogenetic aberrations is in chromosome 9 (aneuploidy, deletions, translocations, inversions), The range of phenotypic consequences found to be associated with these abnormalities are mild growth retardation, malformations of the skull and facial (craniofacial) region, abnormalities of the hands and fingers, skeletal malformations, and/or cardiac defects. Congenital heart anomalies constitute a major malformation leading to significant morbidity and mortality eventually affecting the clinical outcome of the affected individuals. There are several reports of CVDs associated with chromosomal variations like trisomies, deletions, duplications and translocations [22 -24].

The *APO E* gene harbours several polymorphisms, which might be associated with lipoprotein metabolism, obesity, and cardiovascular risk [25, 26]. Chromosome 2q36–37 harbours the insulin receptor substrate-1 (IRS-1) and the calpain-10 (CAPN-10) genes, which are promising candidate genes for both CVD38 and metabolic abnormalities [27].

However, there are no reports of association of pericentric inversion of chromosome 9 [inv(9)(p11-q13)] with CVDs. In the general population, the incidence of pericentric inversion is very frequent and it’s inherited in the Mendelian fashion or without any phenotypic link, this may appear in the child for the first time. This pericentric inversion of

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chromosome 9 and associated mutations have resulted in the occurrence of CVDs in both probands. In support of this, there are reports about abnormalities of chromosome 9 associated with several cardiac anomalies which include truncus arteriosus, truncal valve stenosis, single carotid trunk, subclavian arteries arising from the distal part of the aortic arch, atrial and ventricular septal defects, atrioventricular septal defect, pulmonary atresia, right ventricular hypertrophy and hypoplastic left pulmonary artery. In particular, the chromosome 9p is found to be associated with series of CVDs, like ventricular septal defect with pulmonary valve stenosis and marked hypoplasia of the pulmonary trunk. Although, development of the heart involves a cascade of events involving several genes on different chromosomes, which includes Nkx2-5, Gata4, Tbx5 etc, studies on the molecular level to unravel the gene/s on chromosome 9 which might contribute to the development of the heart, have limited success. Variations in the chromosome 9p21.3 regions one of the strongest associated with a risk factor of CVD, a prospective study of healthy middle-aged men, confirms that the results which were similarly reported from 4 large GWA studies [28].

The *APO E E4* allele has received attention as a genetic risk factor and increase of developing AD. The pathology of the late-onset AD is unknown and the majority of the cases are sporadic and heterogeneous. The *APO E E4* allele has received attention as a genetic risk factor and increase of developing AD. The pathology of the late-onset AD is unknown and the majority of the cases are sporadic and heterogeneous. In the existing study, the *APO E* genotype frequencies of AD (*APO E3/4*= 10%) and CVD (*APO E3/3* = 40 %) were significantly different from control patients. Most of the carriers with *APO E E4* herald the age-related changes seen in the cerebrospinal fluid phosphorylated tau, total tau and amyloid-β1-42 [29]

Corder *et al*. [30] showed an increased frequency of the *APO E E4* allele in the persons with AD when compared with the controls. Similarly, Chandra *et al.* [31] studied *APO E* polymorphisms on Indian cases reported subsidiary higher *E4* frequencies in the subjects aged 55 years or older. Not all persons with *APO E E4* alleles will develop AD nor do all persons with AD carry an *APO E E4* allele. Therefore, *APO E* is neither necessary nor sufficient for the development of AD. There is also an increased risk of developing AD when associated with the increased copies of the *APO E E4* allele. However, the relatively low frequency of the allele in the general and AD populations limits the utility of *APO E*. Earlier studies have reported the association of *APO E E4* allele for both late-onset familial AD and sporadic AD [32-37].

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CONCLUSION

These results are particularly intriguing, given the growing evidence of an association between CVD risk and AD. Considering the early findings, it was found that CVD has a strong genetical background. Genetic risk scores promise to increase understanding of the genetic basis of CVD and improve identification of individuals at increased CVD risk. Identification of chromosomal alterations adds another intriguing dimension and a potential therapeutic target to the complex, clinical elusive and clinically challenging entity in the patients. Carriers harbouring *APO E E4/E4* mutations are at higher risk of disease and those with the *APO E E3/E4* lowest risk, who represent the bulk of families with Alzheimer's disease, typically harbour gene mutation.

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