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Mutational Analysis in Mitochondrial Calcium Uniporter Gene from Patients with Alzheimer's disease

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ABSTRACT Due to population growth total number of people getting affected with Alzheimer's disease (AD) has dramatically increased. Unfortunately, there is no comprehensive analysis of diagnosis, progression, or treatment for AD in India. The chief study objective was to examine the risk factors associated with demographic and clinical features that could contribute to AD in India. A retrospective study was conducted with 174 AD patients with a structured questionnaire. It was observed by the researchers highly educated people from urban area are majorly diagnosed with AD. Cardiovascular diseases were found to be significant among the AD affected individuals. *De novo* point mutations were also identified among the participants. These findings indicate a necessity for proper diagnosis of AD and its follow-up care. It is essential to identify the demographic profile of AD in India to better the living conditions and ensure their proper treatment.

INTRODUCTION

Alzheimer's disease (AD) is one of the neurodegenerative diseases that roots significant cognitive impairment, physical disability, and a significant economic and emotional burden to the affected person, caretaker and society (Peng et al. 2016). The most common form of dementia is AD contributing to about 70 percent of the cases among the elderly. The annual report 2020 by Alzheimer's Association indicates that 3 percent of individuals between the ages of 65 and 74, 17 percent of individuals between the ages of 75 to 84, and 32 percent of individuals above the age of 85 have AD (Ravindranath and Sundarakumar 2021). Ageing is considered a risk factor for neurodegenerative diseases (Venkatesan et al. 2020). The disease is believed to begin 20 years or more before initial symptoms appear, with undetectable changes in the brain of the affected individual. Neurons responsible for basic physical tasks like walking and swallowing are damaged as the disease advances resulting in the individuals becoming bedridden and ultimately a fatal end (Alzheimer's Association 2018). Along with ageing, AD is usually accompanied by other comorbidities as well (Venkatesan et al. 2022). Hence, it is essential to identify potential risk factors that play a significant role in AD (Venkatesan et al. 2021a; Venkatesan et al. 2021b).

The aetiology of AD has been the subject of decades of investigation, yet it remains elusive. When it was shown that excessive calcium intake by the permeability transition pore caused a sub-

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sequent process that resulted in cellular death, a widespread attention in calcium connection with AD pathogenesis began (Rasola and Bernardi 2007; Venugopal et al. 2020). One of the most discriminating transporter complexes located in the inner mitochondrial membrane, the mitochondrial calcium uniporter (MCU), is essential for calcium signalling. MICU1 (mitochondrial calcium uptake protein 1) is a subunit in the MCU complex and controls calcium influx by activating the MCU subunit. According to studies, both in mice and humans, the loss of MICU1 results in calcium building up in the mitochondria, which causes neuronal abnormalities and disorders of muscle function (Liao et al. 2017).

Awareness of AD is low in India and its onset is often associated with old age causing a delay in their diagnosis and treatment. Hence it is imperative to hold a nation-wide awareness campaign for the family members and caregivers. Research on AD is negligible in India and most of the available data are from regional studies. It is necessary to do a pan India census to identify the current incidence and burden of AD in India. The quality of life for the affected individuals was severely influenced as a result of socioeconomic factors and coexistence of other medical disorders, all of which might play a role in the increase of prevalence and mortality of dementia. Besides the above-mentioned factors, the affected individual's mental disorder gives additional stress to the afflicted person and their caretakers, further reducing their quality of life and necessitating the need for institutionalisation (Jelastopulu et al. 2014). The aim of this research was to collect information in order to evaluate the demographic profile of AD patients in South India and to identify potential risk factors.

METHODOLOGY

Subject Recruitment

Participants were selected with the guidance of medical doctors from reputed hospitals and rehabilitation centres from 2017 to 2019. Tamil Nadu and Kerala were the places selected for this study. The participants had a documented diagnosis of AD from at least 2 certified medical representatives to confirm the diagnostic validity. The cohort comprised a total of 174 participants belonging to two groups (n = 174). The study cohort consisted of early onset dementia (aged less than 65, n = 78) and late onset dementia groups (aged more than 65, n = 96). The required consent to take part in the research was obtained from the volunteering individuals' guardian or a well-wisher. The protocols were submitted, and ethical approval was granted by the institutional ethical review board committee of Bharathiar University, Coimbatore, Tamil Nadu, India. The ethical clearance is based on guidelines from the Indian Council of Medical Research (ICMR) and the Declaration of Helsinki (2000) by the World Medical Association (WMA).

Questionnaire

Questionnaires for the cognitive assessment in dementia were done according to the Mini-Mental State Exam (MMSE). This test is a 30-point test where if the individual scores between 24 and 30, it indicates that there is no cognitive impairment. If the score lies between 18 and 23, there is mild cognitive impairment and scores between 0 and 17 indicate severe cognitive impairment. The MMSE test is easy and quick to perform with no additional equipment requirement.

Inclusion and Exclusion Factors

The study included participants with co-morbidities related to AD such as hypertension, diabetes, stroke and coronary heart disease and were properly documented. The healthy controls were not affected by any comorbidities.

The study excluded participants with other forms of dementia. Also, participants with a history of psychosis, substance related disorder and traumatic head injury were excluded for this study.

Genomic DNA Extraction

Fresh blood samples were collected from peripheral blood of participants post obtaining approval from families. Genomic DNA was isolated from blood that had been collected in EDTA-containing tubes using standard protocols. The yield and purity of DNA isolated from the participants was calculated using spectrophotometric analysis.

DNA Amplification

Primer was designed for a coding exon of *MICU1* using Primer 3. DNA fragment for the corresponding primer was amplified using thermocycler (Veriti 96 – well Thermal Cycler, Applied Biosystems) with thermocycling conditions - 95°C x 1 minute (initial denaturation for 1 cycle), 95°C x 30 seconds, 60°C x 45 seconds, 72°C x 7 minutes (35 cycles). The samples were amplified in triplicates.

Mutational Analysis

The amplified DNA was sequenced using dideoxy chain termination method on a 3500 Genetic Analyzer (Applied Biosystems). The sequences were then analysed using DNA Baser Sequence Assembly software. Differences in bases among patient chromatograms and reference chromatograms were analysed and compared to previously reported mutations or polymorphisms. The samples were analysed in triplicates.

Statistics

Data was analysed with the help of Statistical Package for Social Sciences software (SPSS v.1.0. 0.1275). Sociodemographic differences among the patients were calculated using Chi square test and t-tests. The differences in comorbidities were also analysed using Chi-square test and t-tests. For statistical significance, a two-sided p-value of 0.05 or less was taken into account.

RESULTS

In the present study, a total of 174 subjects were recruited with the same number of controls who were age and sex matched. The participants were classified into three categories, that is, mild, moderate and severe. The case group comprised a total of 85 males of which 33 percent belonged to mild AD, 45.9 percent belonged to moderate AD and 21.2 percent belonged to severe AD. In case of females, a total of 89 females were split into 16.9 percent in mild AD, 52.8 percent in moderate AD and 30.3 percent in severe AD (Table 1). The early onset group had an age range of 35-65 years while the late onset age range was between 66-89 years.

Of the 174 participants, only 4 individuals (belonging to the moderate AD group) had a minimal education till high school while the remaining participants were at least graduates. Again, among the remaining 170 participants, 39 individuals were graduates belonging to all three categories (18% in mild AD, 28.2% in moderate AD and 53.8% in severe AD groups, respectively). Surprisingly, a higher number of participants (n=131) were postgraduates and a majority belonged to the active workforce members before the sudden onset of AD. The postgraduate participants were categorised as 27.5 percent in mild AD, 54.2 percent in moderate AD and 18.3 percent in severe AD groups, respectively. Another interesting fact noticed was that a majority of the participants (n=148) were from urban residences as opposed to (n=26) belonging to the rural regions (Table 1).

Table 1: Demographic characteristics of AD participants

Characteristics	*Number of participants (n= 174)*	*Mild AD (n= 43)*	*Moderate AD (n= 86)*	*Severe AD(n= 45)*
Gender				
Male	85	28 (33%)	39 (45.9%)	18 (21.2%)
Female	89	15 (16.9 %)	47 (52.8%)	27 (30.3%)
Group				
Early onset	78	18 (23.1%)	38 (48.7%)	22 (28.2%)
Late onset	96	25 (26.0%)	48 (50.0%)	23 (24.0%)
Education				
High School	4	-	4 (100%)	-
Graduation	39	7 (18%)	11 (28.2%)	21 (53.8%)
Post-graduation	131	36 (27.5%)	71 (54.2%)	24 (18.3%)
Residence				
Rural	26	-	11 (42.3%)	15 (57.7%)
Urban	148	43 (29.1%)	75 (50.7%)	30 (20.3%)

With the assistance from the questionnaire, potential risk factors for these AD participants were also calculated. The most common comorbidities listed were cardiovascular issues, diabetes, hypertension, physical inactiveness, smoking, alcohol and stroke. Among all the three categories, hypertension ranked high (37.2% in mild AD, 43.5% in moderate AD and 41.9% in severe AD groups, respectively) though it proved to be insignificant ($p > 0.05$). This ranking was followed by physical inactiveness, diabetes, smoking, alcohol, stroke and cardiovascular diseases. Statistical analysis revealed a significant $p < 0.05$ in the case of only cardiovascular related diseases (Table 2). Mutational analysis of *MICU1* gene in AD participants was also performed. The analysis led to the identification of three-point mutations of which two were synonymous variants and one was a missense variant (Table 3). The variants were identified in three different loci of the gene.

DISCUSSION

Mortality rates among the elderly have been dropping due to advanced medical interventions, resulting in an increase in the proportion of elderly people around the world, particularly in major cities. Due to the scarcity of epidemiological data in India, this study helps in adding to the understanding of the epidemiology of AD in the country. A few studies have previously identified age and educational level as risk factors for AD (Nunes et al.2010) while other large cohort studies have shown that there are no such risks (Chêne et al. 2015; Horst et al. 2021). Similar to the large cohort studies, the present study also shows no differences between genders and age, which implies that these factors may not be necessarily risk factors for AD (Table 1).

The study indicated that cardiovascular diseases were highly significant for the individuals with AD (Table 2). But this was not supported by earlier studies carried out in India. To lessen the burden of AD and comorbidities, the foregoing result highlights the necessity for focused public health interventions throughout their lives. Factors such as old age, education level, residential areas, physical inactivity, smoking and alcohol have all been shown to be associated with cognition (Ravindranath and Sundarakumar 2021; Alsumari et al. 2019). Stroke has become increasingly prevalent in India in recent years and is growing as a major risk factor of AD (Ravindranath and Sundarakumar 2021). It is necessary to identify the major risk factors for AD and pinpoint potential biomarkers, which are exclusive for AD (Jayaramayya et al. 2020; Devi et al. 2020).

In this study, mutational analysis of *MICU1* has revealed *de novo* point mutations in three regions (Table 3) with little to no effect on the result-

Table 2: Potential risk factors of AD participants

Characteristics	*Mild AD*	*Moderate AD*	*Severe AD*	*p value*
Cardiovascular	6.7%	3.3%	4.2%	0.005
Diabetes	23.0%	29.6%	34.1%	0.165
Hypertension	37.2%	43.5%	41.9%	0.304
Physical inactiveness	26.5%	28.4%	47.3%	0.198
Smoking	17.1%	10.2%	4.3%	0.507
Alcohol	13.9%	7.7%	3.8%	0.419
Stroke	6.3%	5.7%	3.1%	0.238

Table 3: Sequence analysis of AD participants

S. No.	*Transcript*	*Location: bp*	*Alleles*	*Class*	*Variant type*	*Type of mutation*	*Amino acid residue*
1.	ENST00000338518.8	11:70472872	T/C	SNP	Synonymous variant	Point mutation	Arg
2.	ENST0000033850.8	Mt: 8927	C/T	SNP	Missense variant	Point mutation	Met/Ile
3.	ENST00000361381.2	Mt: 10771	A/G	SNP	Synonymous variant	Point mutation	Leu

ing functional protein. According to studies, the loss of *MICU1* results in calcium building up in the mitochondria, which causes neuronal abnormalities and disorders of muscle function, in mice and humans (Liu et al. 2016; Antony et al. 2016). As one ages, mitochondrial viability has been proven to decline. This might be a cause for the natural reduction in calcium uptake by MCU as people age, which explains why AD is more prone in people as they get older.

CONCLUSION

The prevalence of AD in India is anticipated to rise drastically. Due to lack of awareness, diagnosis of AD is not efficient. This study has shown cardiovascular disease as a significant comorbidity of AD but further large cohort studies must be carried out before it can be considered a risk factor. Furthermore, the study showed three *de novo* mutations in *MICU1*. It is necessary to carry out mutational studies in large populations to understand the implications of the gene in AD.

RECOMMENDATIONS

Though AD is prevalent in India, there is still an absence of epidemiological data. It is highly recommended to spread knowledge regarding AD to the community through awareness camps. There is an urgent need to increase awareness and improve the diagnosis and understanding of AD. Recognizing the complex interactions by which these risk variables relate to the aetiopathogenesis of AD may help in identifying the risk factors and distinguishing between AD that develops early (before age 60) and dementia that develops late (after age 60).

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AUTHOR CONTRIBUTIONS

Conceptualisation: A.V., B.V.; resources and data curation: A.V., A.E., H.W.S.B., N.S., A.D.T., S.S.S.; writing and original draft preparation: A.V.; writing, review and editing: B.G., A.N., B.V.; supervision: B.V.

CONFLICT OF INTEREST

The authors declare there are no conflicts of interest.

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