DOI: 10.7860/JCDR/2021/50680.15640

Original Article

Genetics Section

Role of Apolipoprotein E Gene Variants in Cognitive Outcome of Patients after Moderate Degree Diffuse Brain Injuries

MOMIN ABDUL JALEEL[1], SYEDA ZUBEDA[2], ADITYA GRANDHI[3], QURRATULAIN HASAN[4]

ABSTRACT

Introduction: Diffuse Brain Injury (DBI) has been shown to be the major form of the primary brain traumatic injury. Apolipoprotein E (APOE) is considered to be associated in the prognosis of DBI.

Aim: To analyse change in cognitive outcome of moderate degree DBI patients and its association with APOE gene polymorphism.

Materials and Methods: This prospective cohort study was conducted at the Department of Genetics and Molecular Medicine, Kamineni Hospitals, LB Nagar, Hyderabad, Telangana, India, from March 2018 to May 2019, in which 23 patients who had moderate DBI were included. Patients were genotyped for APOE polymorphism and intellectual function was assessed when the patient was being discharged using Mini Mental State Examination (MMSE) score and was repeated after three months. Wilcoxon Signed Rank Test was used to determine the change in MMSE score taken at the time of discharge and after three months in follow-up.

Results: Of the total 23 patients, 19 (82.60) were males and 4 (17.40%) were females where the average age of subjects included was 41.59±17.64 years. E2/E3 APOE genotype was present in 16 (69.56%) subjects. The improvement of MMSE score at the end of three months following discharge showed that best recovery was in subjects with E2/E3, genotype with mean of 4.9±2.1, followed by E2/E2 i.e., 3.5±2.6. In order to evaluate the changes in MMSE score at the time of discharge and after three months due to the presence of APOE genotype E2/E3 a Wilcoxon Signed Rank Test was performed which revealed statistically significant positive change in MMSE score after three months, z=-3.512, p=0.00044, with a large effect size (r=0.620).

Conclusion: This preliminary study indicated that APOE E2/E3 individuals showed a better recovery after traumatic brain injuries based on MMSE scores. However, extended studies are needed to establish this which may also give more weightage for genotyping APOE as a prognostic marker.

Keywords: Genotyping, Mini mental status examination, Neurology, Polymorphism, Prognostic marker

INTRODUCTION

The Diffuse Brain Injury (DBI) has been shown to be the major form of the primary traumatic brain injury. Stritch, in 1956, was the first person to recognise DBI in autopsies of patients with closed brain injuries [1]. DBI is the term applied to induced scattered axonal injury, where tissue damage can be extensive with the involvement of multiple brain areas involving several functional systems. DBI represents a primary, diffuse post-traumatic effect, which is encountered in more than 50% of severe intra-axial traumatic brain injuries and generates extended microscopic lesions of the white matter tracts. Rotational acceleration produces DBI, which is linked with extensive brain dysfunction often without macroscopic damage [1].

These injuries are responsible for the loss of consciousness lasting for more than 6 hours, brainstem signs, and cognitive deficiencies. The important functional neuropsychological changes which may be affected are the speed at which the information is being processed, the amount of attention being paid and the level of concentration, the totality of things remembered like memory and the proper functioning of frontal portion [2].

In Traumatic Brain Injuries (TBI), there is physiological alteration of the functions of brain which demonstrates, a short duration of unconsciousness, episodes of seizures, loss of memory of the incidents that occurred prior and subsequent to the accident. The severity of injury is measured by Glasgow Coma Scale (GCS) which is calculated based on: a) Eye response, which has a score 1-4; (b) Verbal response which has a score of 1-5; (c) Motor response which has a score of 1-6 [3]. Grading of head injuries is based on GCS score. GCS 14 and 15 are treated as mild head injuries, GCS score of 9-13 are treated as moderate head injuries and GCS score of 8 and less are treated as severe head injuries [2].

It has been suggested that the polymorphism of the APOE gene have shown influence on different outcomes after DBI. APOE is usually found in chylomicrons and intermediate density lipoproteins, primarily produced by liver and macrophages. As a response to stress or injury, it is also produced by astrocytes and glial cells that are present in the Central Nervous System (CNS). The APOE essentially functions in redeployment of cholesterol from cells during neurotic extension, growth, repairs and membrane synthesis [4].

The carriage of cholesterol and lipids are performed by APOE, throughout the body and it is also critically involved in their redeployment to the cells by binding to lipoproteins of various sizes and shapes for repair process in the CNS [5]. Although the three alleles of APOE: APOE2 (Cys112, Cys158), APOE3 (Cys112, Arg158) and APOE4 (Arg112, Arg158) [6] differ from each other in only one amino acid at two residues, it gives rise to a highly polymorphic human APOE protein of 299 amino acids that results in varied molecular and cellular effects on its structure and function, which results in different outcomes in TBI patients [7].

APOE gene is located on chromosome 19. e2, e3, e4 are the different alleles of APOE which code for its three common iso-forms. APOE e2 is also associated with amplified risk of atherosclerosis, e3 is considered as a neutral genotype, while e4 is associated with relative risk for alzheimer's disease. APOE4 is also implicated with weakened cognitive function, as well as, unfavorable outcome after brain injury [8].

During the repair process in response to a neuronal damage APOE is synthesised in the brain and the individuals with APOE4 allele have shown to have neurotoxic fragments due to proteolysis resulting from conformational changes and thus common leading to disease associated mitochondrial dysfunctions and cytoskeletal alterations [9].

The objectives of this study were, firstly to genotype the individuals with traumatic brain injury for APOE e4 allele followed by assessing the mini mental state of the individuals with TBI at the time of discharge and at three months follow-up, which can be a good prognostic marker for TBI patients.

MATERIALS AND METHODS

This prospective cohort study was conducted at the Department of Genetics and Molecular Medicine, Kamineni Hospitals LB Nagar, Hyderabad, Telangana, India, from March 2018 to May 2019. Ethics Committee approval was obtained from the Institutional Ethics Committee of Kamineni Hospitals (Registration Number: ECR/58/Inst/AP/2013/RR-16) Hyderabad, India and an informed consent of the patient/attender was obtained. In this study, the association of different alleles of APOE gene with specific (good/bad/moderate) cognitive outcome of the patient who had a diffuse/traumatic brain injury was analysed.

Sample size calculation: Sample size was calculated by applying the formula

$$n=(Z * \Sigma/E)^2$$

Where,

n=sample size,

Z is a constant and its value is 2.58,

E is error: 5%

and estimated sigma or standard deviation (8.5) was calculated by taking sample sizes from seven different studies [10-16]. The estimated sample size was 20 and hence, 23 patients were included in study.

A total of 23 patients with moderate degree DBI admitted in Kamineni Hospitals, Hyderabad, India, were selected based on either Computed Tomography (CT) scan (Marshall grade I to IV) [17], or Magnetic Resonance Imaging (MRI) scan suggestive of DBI.

Inclusion criteria: All patients with GCS of 9-13 at the time of admission with a radiological evidence of DBI (Marshall grade I to IV) in the age group of 14-70 years were included in the study.

Exclusion criteria: Patients with cognitive impairment before injury like those who had a history of alzheimer's or parkinson's disease or loss of memory or those patients who had previous head injury/stroke/meningitis/encephalitis/or individuals with documented cerebral palsy/any other neurological disease were excluded. Patients with focal lesions like extradural haematoma/subdural haematoma/contusions (>25 mL) were also excluded [14].

Isolation of Genomic DNA

Isolation of genomic Deoxyribonucleic Acid (DNA) was carried out from Ethylenediamine Tetraacetic Acid (EDTA) blood by Salting out method as per our published protocol [12]. Briefly the blood samples were treated with red blood cell lysis buffer, followed by white blood cell lysis buffer along with 10% Sodium Dodecyl Sulphate (SDS) and subsequently 6M sodium chloride (NaCl) was added to precipitate the proteins and the supernatant containing DNA was transferred to another Eppendorf tube with pre chilled isopropanol and subsequently this DNA was subjected to ethanol washes and is dissolved in 50 µL of Tris- EDTA buffer and stored at -20° C freezer until further use.

Polymerase Chain Reaction (PCR) and Restriction Fragment Length Polymorphism (RFLP)

The 1 µL of the isolated DNA was used to set up a PCR reaction of a total volume of 25 µL with 2mM $MgCl^2$ concentration and by using the forward and reverse primer which encompasses the sequence change present in the 4th exon of APOE gene. The sequence of the Forward Primer: 5'-GCCTACAAATCGGAACTGGA-3' and the Reverse Primer: 5'-GCCCCGGCCTGGTACACTGCCA- 3' and these are synthesised commercially at SIGMA, Hyderabad (India).

A three-step amplification reaction using thermal cycler (Gene Mate) was carried out [5]. The PCR settings included one step of initial denaturation for 5 min at 95ºC which makes the double strand DNA uncoil then in subsequent 35 cycles of three steps which includes denaturation at 95ºC for 30 seconds, annealing at 64ºC for 30 seconds wherein the primer anneals to specific location in DNA and extension at 72ºC for 1 minute in which bases are added and final extension at 72ºC for 7 minutes [18].

The so obtained PCR products were then run in an electrophoresis tank by loading in 2% agarose gel and the amplified DNA was visualised as bands using a gel documentation system (Kamineni, Hyd). Double distilled water was used as negative control and a sample with known APOE genotype was used as positive control in each batch of PCR. The obtained PCR products were subjected to restriction digestion with Afl III and Hae II enzymes for 16 hours at 37°C and were analysed on 10% PAGE [19].

Mini Mental State Examination (MMSE) score was carried out at the time of discharge and was repeated after three months, when the patients came for a follow-up visit. MMSE evaluates orientation, registration, attention, calculation, recall and language [3]. The difference between MMSE score calculated just before the time of discharge and three months later indicates recovery. Results of this score were compared with APOE genotypes.

STATISTICAL ANALYSIS

Wilcoxon Signed Rank Test was used to determine the change in MMSE score taken at the time of discharge and after three months in follow-up by using Wilcoxon Signed-Rank Test Calculator available online at https://www.socscistatistics.com. The r (effect size) value was calculated by using the formula $r=Z/\sqrt{N}$, where z=-3.512, n=23 (no. of cases in that group/e2e3 multiplied by 2) and interpreted based on Cohen's 1988 [20]. Microsoft Excel 2013 and GraphPad Prism software version 8.0.2 were used to prepare the data.

RESULTS

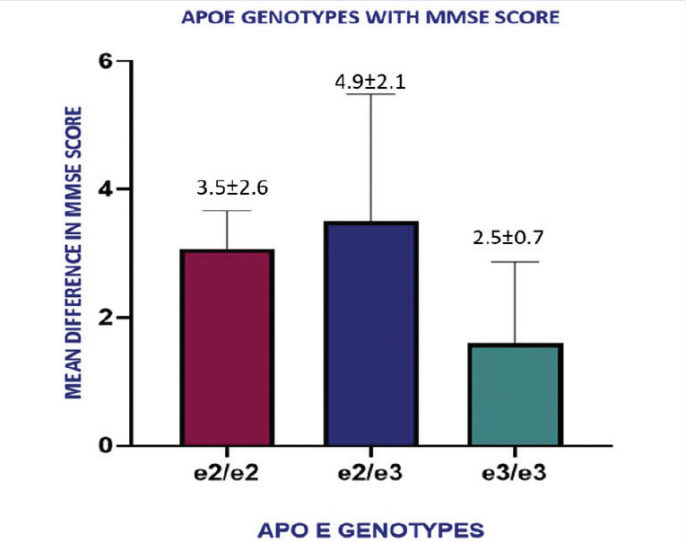
In the preliminary study, a total of 23 subjects were included of which 19 (82.60%) were males and 4 (17.40%) were females. The average age of subjects included was 41.59±17.64 years whereas the mean age of males was 41.27±17.72 years and that of females was 44±24 years. Mode of injury included road traffic accident: 19 (82.60%),, assault: 1 (4.35%), slip and fall: 1 (4.35%) , fall from stairs: 2 (8.70%). A 5 (21.73%) were categorised as Marshall grade I, 10 (43.5%) as Grade II, 7 (30.43%) as grade III and 1 (4.34%) as Grade IV.

APOE alleles were genotyped without being aware of the GCS scores. The individuals with E2/E2 genotype were 4 (17.4%), E2/E3 was 16 (69.56%), E3/E3 was 2 (8.7%) and E3/E4 was 1 (4.34%), patients. APOE genotypes with the respective MMSE scores along with recovery value (difference in MMSE score at discharge and after 3 months) are represented in [Table/Fig-1].

The improvement of MMSE score at the end of three months following discharge showed that best recovery was in subjects with E2/E3 genotype with mean of 4.9±2.1, followed by E2/E2 i.e., 3.5±2.6 [Table/Fig-2]. In order to evaluate the changes in MMSE score at the time of discharge and after three months due to the presence of APOE genotype E2/E3 a Wilcoxon Signed Rank Test was performed which revealed statistically significant positive change in MMSE score after three months, z=-3.512, p-value=0.00044, with a large effect size (r=0.620). r=0.620, indicating a large effect size using Cohen (1988) criteria which states 0.1=small effect, 0.3=medium effect, 0.5=large effect.

Patients	APOE genotype	MMSE immediate	MMSE after 3 months	Difference
1.	E2/E2	25	28	3
2.	E2/E2	22	28	6
3.	E2/E2	30	30	0
4.	E2/E2	23	28	5
5.	E2/E3	16	25	9
6.	E2/E3	4	11	7
7.	E2/E3	29	30	1
8.	E2/E3	12	20	8
9.	E2/E3	23	27	4
10.	E2/E3	23	28	5
11.	E2/E3	24	27	3
12.	E2/E3	24	28	4
13.	E2/E3	23	26	3
14.	E3/E3	21	23	2
15.	E3/E3	19	27	3
16.	E3/E4	17	26	9
17.	E2/E3	22	28	6
18.	E2/E3	20	25	5
19.	E2/E3	21	27	6
20.	E2/E3	25	28	3
21.	E2/E3	24	27	3
22.	E2/E3	21	27	6
23.	E2/E3	20	26	6

[Table/Fig-1]: The APOE genotypes along with Mini Mental State Examination (MMSE) score of 23 patients at the time of discharge and after three months accompanied with the difference in their respective before and after MMSE scores.



[Table/Fig-2]: Mean MMSE score with standard deviation in different APOE genotypes.

DISCUSSION

Response to the brain injury and following developments in repair is evidently associated with multifunctional APOE protein [21]. Since, APOE plays a critical role in upholding the cerebral vasculature and the integral structure of the Central Nervous System (CNS), its influence on the repair process after TBI has also been very evident. In one of the studies by Methia N et al., compromised integrity of blood brain barrier was seen which was caused due to lack of APOE protein after brain injury [22].

This study has projected that different APOE genotypes show variance in cognitive outcome of the patients after moderate degree DBI. In this preliminary study, there was one 25-year-old patient with APOE4 allele. His MMSE score after three months showed an improvement by 9 points. As the patient was young (25 years) and a heterozygote carrier of e4 allele, the improvement may also be attributed to either the patient's young age or the other allele which is E3 which can be delivering the normal function of the protein as reported in previous studies. Tesdale GM et al., showed that patients with acute head injury have shown a striking evidence of difference in recovery which is determined by genetic component/alleles and also the difference in the age of the patients [4].

Bad outcome was apparent in approximately double dose in individuals with APOE4 allele in a study by Tesdale GM et al., whereas, some studies reported very negligible or equivalent to no association of outcome with APOE4 [4,23,24]. The presence of very huge volume of intracranial haematomas in APOE4 carriers after TBI with diminished cerebrovascular integrity has portrayed a straight indication of their association [25].

Yue JK et al., and Anderson GD et al., showed that e4 carriers show association with delay free recall and memory compared to non e4 carriers. Similarly in this study, non e4 carrier i.e., e3 and e2 genotypes showed recovery in MMSE score. However, e3 carriers showed maximum recovery [26,27]. Even though, study by Noe E et al., and Chirivella J et al., showed an association in the downstream cognitive recovery in TBI patients with APOE, a study by Vanderploeg RD et al., reported not much change was seen in short and long cued recall between subjects succumbed to TBI when compared with controls [28,29].

Crawford FC et al., have shown that even though the severity of TBI was similar in subjects expressing APOE4, e2 or e3 allele, individuals who possessed APOE4 allele faced comparatively more difficulties in verbal memory and fluency than the latter, in follow-up (after 6 months post TBI) check-ups [30]. Although, Chamelian L et al., stated that APOE4 individuals who had good verbal intellectual and verbal memory skills pre injury did not perform the same post TBI, these differences on multiple assessments may not be equally significant [24].

In this prospective study, on traumatic brain injuries it was also observed that the MMSE score is better after three months in all patients, but the improvement is best in individuals with E2/E3 genotype, this may be due to APOE levels in CNS, as it has been reported by Flowers SA et al., and others that the presence of APOE protein is higher in the CNS of individuals who have APOE3 compared to those with APOE4 [31-34].

Limitation(s)

The number of samples included in the study was relatively less. Larger studies with different subjects including various ethnic groups are required for stronger evidence.

CONCLUSION(S)

This preliminary study indicated that APO E2/E3 individuals showed a better recovery after traumatic brain injury based on MMSE scores. Genotyping of APOE polymorphism in traumatic head injury patients can predict the probable outcome of the patient and a better understanding of one of the cause behind such, good or bad outcome. However, extended studies are needed to establish this which may also give more weightage for genotyping APOE as a prognostic marker.

Acknowledgement

Authors would like to thank Kamineni Academy of Medical Sciences.

REFERENCES

[1] Pearce JM. Observations on concussion. A review. Eur Neurol. 2008;59(3-4):113-19.
[2] Teasdale G, Jennett B. Assessment of coma and impaired consciousness. A practical scale. Lancet. 1974;2(7872):81-84.
[3] Middleton PM. Practical use of the Glasgow Coma Scale; a comprehensive narrative review of GCS methodology. Australas Emerg Nurs J. 2012;15(3):170-83.
[4] Teasdale GM, Murray GD, Nicoll JA. The association between APOE epsilon4, age and outcome after head injury: A prospective cohort study. Brain. 2005;128(Pt 11):2556-61.

[5] Achariyar TM, Li B, Peng W, Verghese PB, Shi Y, McConnell E, et al. Glymphatic distribution of CSF-derived apoE into brain is isoform specific and suppressed during sleep deprivation. Mol Neurodegener. 2016;11(1):74.
[6] Rall SC Jr, Weisgraber KH, Mahley RW. Human apolipoprotein E. The complete amino acid sequence. J Biol Chem. 1982;257(8):4171-78.
[7] Mahley RW, Rall SC Jr. Apolipoprotein E: Far more than a lipid transport protein. Annu Rev Genomics Hum Genet. 2000;1:507-37.
[8] Verghese PB, Castellano JM, Holtzman DM. Apolipoprotein E in Alzheimer's disease and other neurological disorders. Lancet Neurol. 2011;10(3):241-52.
[9] Mahley RW, Huang Y. Apolipoprotein e sets the stage: Response to injury triggers neuropathology. Neuron. 2012;76(5):871-85.
[10] Jordan BD, Relkin NR, Ravdin LD, Jacobs AR, Bennett A, Gandy S. Apolipoprotein E e4 associated with chronic traumatic brain injury in boxing. JAMA. 1997;278:136-40.
[11] Kutner KC, Erlanger DM, Tsai J, Jordan B, Relkin NR. Lower cognitive performance of older football players possessing apolipoprotein E e4. Neurosurgery. 2000;47:651-57; discussion 657-658.
[12] Johnson LW, Lundgren K, Henrich V, Phillips S. Factors influencing recovery from mild traumatic brain injury. Brain Inj. 2020;34(9):1202-12.
[13] Muller K, Ingebrigtsen T, Wilsgaard T, Wikran G, Fagerheim T, Romner B, et al. Prediction of time trends in recovery of cognitive function after mild head injury. Neurosurgery. 2009;64:698-704.
[14] Shadli RM, Pieter MS, Yaacob MJ, Rashid FA. APOE genotype and neuropsychological outcome in mild-to-moderate traumatic brain injury: A pilot study. Brain Inj. 2011;25:596-603.
[15] Hiekkanen H, Kurki T, Brandstack N, Kairisto V, Tenovuo O. Association of injury severity, MRI-results and ApoE genotype with 1-year outcome in mainly mild TBI: A preliminary study. Brain Inj. 2009;23:396-402.
[16] Lichtman SW, Seliger G, Tycko B, Marder K. Apolipoprotein E and functional recovery from brain injury following postacute rehabilitation. Neurology. 2000;55:1536-39.
[17] Marshall LF, Marshall SB, Klauber MR, Clark MB, Eisenberg HM, Jane JA, et al. A new classification of head injury based on computerized tomography. Special Supplements. 1991;75:S14-20.
[18] Daram S, Syeda Z, Poornima S, Boppana S, Prabhala S, Sandhya A, et al. Association of Androgen Receptor (AR) CAG Repeats and Cytochrome P450 3A5*3 (CYP3A5*3) Gene Polymorphisms in South Indian Men with Prostate Cancer. J Clin Diagn Res. 2019;13(8):GC01-04.
[19] Thathapudi S, Kodati V, Erukkambattu J, Katragadda A, Addepally U, Hasan Q. Tumor necrosis factor-alpha and polycystic ovarian syndrome: A clinical, biochemical, and molecular genetic study. Genet Test Mol Biomarkers. 2014;18(9):605-09.
[20] Lakens D. Calculating and reporting effect sizes to facilitate cumulative science: A practical primer for t-tests and ANOVAs. Front Psychol. 2013;4:863.
[21] Horsburgh K, McCarron MO, White F, Nicoll JA. The role of apolipoprotein E in Alzheimer's disease, acute brain injury and cerebrovascular disease: Evidence of common mechanisms and utility of animal models. Neurobiol Aging. 2000;21(2):245-55.
[22] Methia N, André P, Hafezi-Moghadam A, Economopoulos M, Thomas KL, Wagner DD. ApoE deficiency compromises the blood brain barrier especially after injury. Mol Med. 2001;7(12):810-15.
[23] Sundström A, Marklund P, Nilsson LG, Cruts M, Adolfsson R, Van Broeckhoven C, et al. APOE influences on neuropsychological function after mild head injury: Within-person comparisons. Neurology. 2004;62(11):1963-66.
[24] Chamelian L, Reis M, Feinstein A. Six-month recovery from mild to moderate Traumatic Brain Injury: The role of APOE-epsilon4 allele. Brain. 2004;127(Pt 12):2621-28.
[25] Liaquat I, Dunn LT, Nicoll JA, Teasdale GM, Norrie JD. Effect of apolipoprotein E genotype on hematoma volume after trauma. J Neurosurg. 2002;96(1):90-96.
[26] Yue JK, Robinson CK, Burke JF, Winkler EA, Deng H, Cnossen MC, et al. Apolipoprotein E epsilon 4 (APOE-ε4) genotype is associated with decreased 6-month verbal memory performance after mild traumatic brain injury. Brain Behav. 2017;7(9):e00791.
[27] Anderson GD, Temkin NR, Dikmen SS, Diaz-Arrastia R, Machamer JE, Farhrenbruch C, et al. Haptoglobin phenotype and apolipoprotein E polymorphism: Relationship to posttraumatic seizures and neuropsychological functioning after traumatic brain injury. Epilepsy Behav. 2009;16(3):501-06.
[28] Noe E, Ferri J, Colomer C, Moliner B, Chirivella J. APOE genotype and verbal memory recovery during and after emergence from post-traumatic amnesia. Brain Inj. 2010;24(6):886-92.
[29] Vanderploeg RD, Crowell TA, Curtiss G. Verbal learning and memory deficits in traumatic brain injury: Encoding, consolidation, and retrieval. J Clin Exp Neuropsychol. 2001;23(2):185-95.
[30] Crawford FC, Vanderploeg RD, Freeman MJ, Singh S, Waisman M, Michaels L, et al. APOE genotype influences acquisition and recall following traumatic brain injury. Neurology. 2002;58(7):1115-18.
[31] Flowers SA, Rebeck GW. APOE in the normal brain. Neurobiol Dis. 2020;136:104724.
[32] Riddell DR, Zhou H, Atchison K, Warwick HK, Atkinson PJ, Jefferson J, et al. Impact of apolipoprotein E (ApoE) polymorphism on brain ApoE levels. J Neurosci. 2008;28(45):11445-53.
[33] Sullivan PM, Han B, Liu F, Mace BE, Ervin JF, Wu S, et al. Reduced levels of human apoE4 protein in an animal model of cognitive impairment. Neurobiol Aging. 2011;32(5):791-801.
[34] Vitek MP, Brown CM, Colton CA. APOE genotype-specific differences in the innate immune response. Neurobiol Aging. 2009;30(9):1350-60.

PARTICULARS OF CONTRIBUTORS:
1. Associate Professor, Department of Neurosurgery, Kamineni Hospitals, L.B Nagar, Hyderabad, Telangana, India.
2. Research Officer, Department of Genetics and Molecular Medicine, Kamineni Hospitals, L.B Nagar, Hyderabad, Telangana, India.
3. DNB, Department of Neurosurgery, Kamineni Hospitals, L.B Nagar, Hyderabad, Telangana, India.
4. Head, Department of Genetics and Molecular Medicine, Kamineni Hospitals, L.B Nagar, Hyderabad, Telangana, India.

NAME, ADDRESS, E-MAIL ID OF THE CORRESPONDING AUTHOR:
Dr. Qurratulain Hasan,
Head, Department of Genetics and Molecular Medicine, Kamineni Hospitals, L.B Nagar, Hyderabad, Telangana, India.
E-mail: qhasan2013@gmail.com

PLAGIARISM CHECKING METHODS: [Jain H et al.]
- Plagiarism X-checker: Jun 04, 2021
- Manual Googling: Sep 15, 2021
- iThenticate Software: Sep 22, 2021 (8%)

ETYMOLOGY: Author Origin

AUTHOR DECLARATION:
- Financial or Other Competing Interests: Funded by Kamineni Academy of Medical Sciences
- Was Ethics Committee Approval obtained for this study? Yes
- Was informed consent obtained from the subjects involved in the study? Yes
- For any images presented appropriate consent has been obtained from the subjects. No

Date of Submission: **Jun 03, 2021**
Date of Peer Review: **Jun 26, 2021**
Date of Acceptance: **Sep 17, 2021**
Date of Publishing: **Nov 01, 2021**