ROLE OF E121K MUTATION OF D-AMINO ACID OXIDASE IN AMYOTROPHIC LATERAL SCLEROSIS

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by

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CERTIFICATE

This is to certify that the thesis titled "**Role of E121K mutation of D-Amino Acid Oxidase in Amyotrophic Lateral Sclerosis**", submitted by **Ms. Upma Dave** to the Indian Institute of Technology Delhi for the award of the degree of "**Doctor of Philosophy**" is a record of the bonafide research she conducted, which has been prepared under my supervision and guidance following the rules and regulations of the Indian Institute of Technology Delhi, India. The results prescribed in it have not been submitted in part or whole to any other University for the award of any degree or diploma.

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ABSTRACT

Amyotrophic lateral sclerosis (ALS) is a terminal, adult-onset neuromuscular disorder. Our group has been studying this illness and has previously reported a novel E121K mutation in DAO mutations in a study using next-generation sequencing of DNA samples from Indian ALS patients. However, variations in molecular mechanisms caused by this mutation which leads to ALS have not been studied. This report focuses on the computational, biophysical, and cellular study of ALS-associated E121K variant in the human D-amino acid oxidase (*DAO*) to understand the disease pathogenesis mechanisms. Mutations in the *DAO* gene, which encodes the D-amino acid degrading enzyme, have been associated with ALS. DAO is a peroxisomal enzyme containing FAD as a co-factor, and it reacts with D-amino acid donors by targeting the CH-NH2 group. Dysfunction of the DAO protein disrupts D-serine regulation and leads to motor neuron degeneration. Our computational molecular dynamic simulation study results indicate different conformation, activity site loop dynamics, and FAD cofactor binding affinity for E121K DAO. Further to understand this in detail, we conducted comparative biophysical characterization and enzyme activity assay studies of the wildtype- and mutant E121K-DAO. We observed that the purified E121K-DAO was inactive and exhibited a lower affinity for the FAD cofactor and benzoate inhibitor. Structural studies revealed that the E121K mutant has higher beta-sheet content, melting temperature, and oligomeric states than the wildtype. Kinetic study of aggregation of the variants using thioflavin-T confirmed that the E121K-DAO was more prone to aggregation. Microscopic visualization showed that the aggregation proceeds through an intermediate step involving forming fibrillar structures in the E121K mutant. Our results give insights into the pathogenic processes that lead to ALS. Further, our experiments in SH-SY5Y cells proved that E121K mutation causes accumulation of mutant protein aggregates, a change in cell morphology, and the death of neuronal cells. These protein aggregates get ubiquitinated and cause autophagy deregulation in mutant cells. We observed an

increase in the cellular concentrations of p62, OPTN, and LC3II. Through confocal microscopy, we showed that the binding of p62 with ubiquitinated aggregates and its recruitment to LC3II mediates autophagosome generation. These relative changes in the critical partners in autophagy increase cell death in cells harboring the E121K mutation, a probable ALS disease mechanism.

सार

एमियोट्रोफिक लेटरल स्क्लेरोसिस (ALS) एक टर्मिनल, वयस्क-शुरुआत न्यूरोमस्कुलर विकार है। हमारा समूह इस बीमारी का अध्ययन कर रहा है और पहले भारतीय एएलएस रोगियों से अगली पीढ़ी के अनुक्रमण डीएनए नमूनों का उपयोग करते हुए एक अध्ययन में कुछ अन्य नए और दुर्लभ उत्परिवर्तन के साथ डीएओ (DAO) म्यूटेशन में एक नए E121K उत्परिवर्तन की सूचना दी है। हालांकि, इस उत्परिवर्तन के कारण आणविक तंत्र में भिन्नताएं जो एएलएस की ओर ले जाती हैं, का अध्ययन नहीं किया गया है। यह रिपोर्ट रोगजनन तंत्र को समझने के लिए मानव डी-एमिनो एसिड ऑक्सीडेज (DAO) जीन में एएलएस-लिंक्ड E121K उत्परिवर्तन के कम्प्यूटेशनल, बायोफिजिकल और सेलुलर अध्ययन पर केंद्रित है। डीएओ जीन में उत्परिवर्तन, जो डी-एमिनो एसिड नाशक एंजाइम को बनाता है, एएलएस से जुड़ा हुआ है। डीएओ एक पेरॉक्सिसोमल एंजाइम है जिसमें FAD एक सह-कारक के रूप में होता है, और यह डी-अमीनो एसिड दाताओं के CH-NH-2 समूह पर ऑक्सीजन के साथ एक स्वीकार्य के रूप में कार्य करता है। डीएओ प्रोटीन की शिथिलता डी-सेरीन नियमन को बाधित करती है और मोटर न्यूरॉन अध: पतन की ओर ले जाती है। हमारे कम्प्यूटेशनल आणविक गतिशील सिमुलेशन अध्ययन के परिणाम E121K उत्परिवर्ती डीएओ के लिए FAD कॉफ़ैक्टर के विभिन्न रचना, गतिविधि साइट लूप गतिकी और बाध्यकारी आत्मीयता का संकेत देते हैं। इसे और विस्तार से समझने के लिए, हमने वाइल्डटाइप- और उत्परिवर्ती E121K-DAO के तुलनात्मक बायोफिजिकल लक्षण वर्णन

और परख अध्ययन किए। हमने देखा कि शुद्ध E121K-DAO निष्क्रिय था और FAD कॉफ़ैक्टर और बेंजोएट अवरोधक के लिए कम बांधता था। संरचनात्मक अध्ययनों से पता चला है कि E121K उत्परिवर्ती में वाइल्डटाइप प्रकार की तुलना में अधिक बीटा-शीट, पिघलने का तापमान और ओलिगोमेरिक स्थिति हैं। थायोफ्लेविन-टी का उपयोग करने वाले वेरिएंट के एकत्रीकरण के काइनेटिक अध्ययन ने पुष्टि की कि E121K-DAO इकट्ठा के लिए अधिक प्रवण था। माइक्रोस्कोपिक विज़ुअलाइज़ेशन ने दिखाया कि E121K म्यूटेंट में फ़िब्रिलर संरचनाओं को बनाने वाले एक मध्यवर्ती चरण के माध्यम से इकट्ठा आगे बढ़ता है। हमारे परिणाम एएलएस रोगजनन के लिए अग्रणी अंतर्निहित तंत्र में अंतर्दृष्टि प्रदान करते हैं। इसके अलावा, SH-SY5Y कोशिकाओं में हमारे प्रयोगों ने साबित किया कि E121K उत्परिवर्तन के परिणामस्वरूप उत्परिवर्ती प्रोटीन समुच्चय, कोशिका आकृति विज्ञान में परिवर्तन और न्यूरोनल कोशिकाओं की मृत्यु हो जाती है। ये प्रोटीन समुच्चय इकट्ठा हो जाते हैं और ऑटोफैगी विनियमन में असंतुलन पैदा करते हैं। हमने p62, OPTN और LC3II की सेलुलर सांद्रता में वृद्धि देखी। कन्फोकल माइक्रोस्कोपी के माध्यम से, हमने दिखाया कि सर्वव्यापी समुच्चय के साथ p62 का बंधन और LC3II में इसकी भर्ती ऑटोपागोसोम पीढ़ी की मध्यस्थता करती है। ऑटोफैगी में महत्वपूर्ण साझेदारों में ये सापेक्ष परिवर्तन E121K म्यूटेशन, एक संभावित ALS रोग तंत्र को नुकसान पहुँचाने वाली कोशिकाओं में कोशिका मृत्यु को बढ़ाते हैं।

HIGHLIGHTS

The main findings of the study are as follows:

- Molecular dynamic simulation study reveals the distinct conformational state of E121K mutant than Wildtype DAO
- Wildtype DAO has both open and closed active site loop dynamics while E121K DAO mutant is present only in the closed conformation
- Mutant E121K DAO has lower affinity for FAD cofactor than wildtype
- The ALS-associated E121K mutation in the human DAO protein exhibits a complete loss-of-function
- The mutant protein has a reduced ability to bind to the FAD cofactor and benzoate inhibitor
- The E121K variant has higher β-sheet content and oligomeric conformation compared to the wildtype
- The E121K-DAO forms prefibrillar aggregates at physiological pH, which disrupts cellular structure
- The E121K mutation results in the accumulation of mutant protein aggregates and causes a change in SH-SY5Y neuroblastoma cell morphology
- E121K mutation promotes cell death in SH-SY5Y cells.
- The DAO E121K protein is associated with peroxisomal aggregates and ubiquitin binding.
- The DAO E121K protein activates ubiquitin-mediated autophagy and upregulates the expression of p62, OPTN, and LC3II proteins, which subsequently bind with the protein for degradation.

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ACHR	Acetylcholine receptor
ALS	Amyotrophic lateral sclerosis
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid
ANG	Angiogenin
ATXN2	Ataxin 2
BSA	Bovine serum albumin
C9orf72	chromosome 9 open reading frame 72
CCNF	Cyclin F
CD	Circular Dichroism
cDNA	Complementary DNA
CHCHD10	Coiled-coil-helix-coiled-coil-helix domain-containing protein 10
CHMP2B	Charged multivesicular body protein 2B
DAO	D-amino acid oxidase
DAPI	4′,6-diamidino-2-phenylindole
DCTN1	Dynactin subunit 1
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
E. coli	*Escherichia coli*
EC	Enzyme Commission
EGPF	Enhanced green fluorescent protein
ELP3	Elongated acetyltransferase complex subunit 3
EMG	Electromyography
EMG/NCS	Electromyogram and nerve conduction tests

ERBB4	Erb-B2 Receptor Tyrosine Kinase 4
EWSR1	EWS RNA binding protein 1
FAD	flavin adenine dinucleotide
FALS	Familial ALS
FBS	Fetal Bovine Serum
FEL	free energy landscape
FIG4	Factor-induced gene 4
FTD	Frontotemporal dementia
FUS	Fused in sarcoma
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GFP	Green fluorescent protein
Glu/E	Glutamate
GROMACS	GROningen MAchine for Chemical **S**imulations
HCL	Hydrochloride
HNRNP	Heterogeneous nuclear ribonucleoprotein
HRP	Horseradish peroxidase
HSP	Hereditary Spastic Paraplegia
IPTG	Isopropyl β- d-1-thiogalactopyranoside
K_d	Dissociation constant
kDA	Kilo Dalton
LC3-II	Light chain -II
LMN	Lower motor neurons
MATR3	Matrin 3
MD	Molecular Dynamic
MMPBSA	Molecular Mechanics Poisson-Boltzmann Surface Area
mRNA	Messenger RNA
MTT	3-(4, 5-dimethyl thiazolyl-2)-2, 5-diphenyltetrazolium bromide

NaCl	Sodium Chloride
NCS	Nerve conduction studies
NEK1	NIMA-related kinase 1
NMDA	N-methyl-D-aspartate
NTA	Nitrilo triacetic acid
OPMD	Oculopharyngeal muscular dystrophy
OPTN	Optineurin
P62	Sequestosome1
PAGE	Polyacrylamide gel electrophoresis
PCA	Principal component analysis
PCR	Polymerase chain reaction
PDB	Protein Data Bank
PFN1	Profilin 1
PMP70	Peroxisomal marker protein 70
PMSF	Phenyl methyl sulfonyl fluoride
PolyPhen	polymorphism phenotyping
PVDF	Polyvinylidene fluoride
RIPA	Radioimmunoprecipitation
RMSD	Root Mean Square Deviation
RMSF	Root mean square fluctuations
RNA	Ribonucleic acid
RNS	repetitive nerve stimulation
RPM	Rotation per minute
RT-PCR	Reverse Transcription- Polymerase Chain Reaction
S.E.M.	Standard error of the mean
SALS	Sporadic ALS
SASA	Solvent accessible surface area

SBMA	Spinal-Bulbar Muscular Atrophy
SDM	Site-Directed Mutagenesis
SDS	Sodium dodecyl-sulfate
SETX	Senataxin
SIFT	Sorting intolerant from tolerant
SIGMAR1	Sigma non-opioid intracellular receptor 1
SMN	Survival motor neuron
SOD1	superoxide dismutase 1
SPG	Spastacin
SQSTM1	Sequestosome1
TAF15	Tata box binding protein factor associated 15
TBK1	Tank binding kinase 1
TDP43	TAR DNA binding protein 43
TEM	Transmission electron microscopy
ThT	Thioflavin T
T_m	Melting temperature
TUBA4A	Tubulin alpha 4A
UBQLN	Ubiquilin
UBQLN2	Ubiquilin2
UMN	Upper motor neurons
UPS	Ubiquitin-proteasome system
UV	Ultraviolet
VAPB	Vesicle-associated membrane protein B
VCP	Valosin containing protein
VGCC	Voltage-gated calcium channel
WT	Wildtype