FUNCTIONAL INSIGHT INTO THE ROLE OF OPTINEURIN NOVEL VARIANT K489E IN AMYOTROPHIC LATERAL SCLEROSIS (ALS)

DIBYAKANTI MISHRA

KUSUMA SCHOOL OF BIOLOGICAL SCIENCES

INDIAN INSTITUTE OF TECHNOLOGY DELHI

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by

DIBYAKANTI MISHRA

Kusuma School of Biological Sciences

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Dedicated to my divine blue energy…

For his unconditional blessings, love and support

CERTIFICATE

This is to certify that the thesis titled "**Functional insight into the role of optineurin novel variant K489E in Amyotrophic Lateral Sclerosis (ALS)**", submitted by **Ms. Dibyakanti Mishra** 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.

Dr. James Gomes

Professor

Kusuma School of Biological Sciences

Indian Institute of Technology Delhi

New Delhi 110016, India

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Dibyakanti Mishra

ABSTRACT

Mutations in the Optineurin (OPTN) gene have been implicated in amyotrophic lateral sclerosis (ALS). Although optineurin protein (OPTN) is known to regulate autophagy, apoptosis, and other cellular processes, its role in ALS pathology is unclear. Recently, while carrying out a genetic analysis of Indian ALS patients, our group identified a novel mutation K489E in the OPTN gene. Additionally, through whole exome sequencing of blood DNA from 25 Indian ALS patients, we found the same mutation in three Indian ALS patients. This adenine to guanosine missense mutation lies in the ubiquitin-binding domain of OPTN. To identify the molecular mechanism associated with this mutation, we developed an in-vitro cell culture-based model in SH-SY5Y cells bearing the K489E OPTN mutation. We observed that 36.5% more of the K489E mutant cells die compared to the wild type. To ascertain the cause of death in OPTN-K489E cells, we measured the expressions of apoptosis, necroptosis, and autophagy-mediated genes and proteins. We observed alteration in gene expressions of miR-9, REST, CoREST, and BDNF, and their concerted effect on the regulation of miRNA-9-mediated apoptosis. We also observed alterations in the expressions of necroptosis-mediating genes RIPK1, RIPK3, and MLKL compared to the wild type. Our results show upregulation of the expressions of autophagy mediating proteins TBK1, P62, and LC3II. We have also seen the effect of this mutation on its binding partners like TBK1 through immunoprecipitation, confocal microscopy and RT-PCR. In the current scenario, there is no permanent cure for this disease, but researchers are trying continuously to delay the progression of this disease by studying various drugs and vitamins. Similarly, we have also studied the effect of vitamin C and vitamin D on the mutant model of SH-SY5Y. We found an increase in cell survival in the case of the mutant after the addition of vitamin C. We have also focused on how this cell survival is happening in OPTN-K489E-expressing cells.

सार

ऑप्टिन्यूरिन (ओपीटीएन) जीन में उत्परिवर्तन को एमियोट्रोफिक लेटरल स्क्लेरोसिस (एएलएस) में शामिल किया गया है। यद्यपि ऑप्टिनेरिन प्रोटीन (ओपीटीएन) को ऑटोफैगी, एपोप्टोसिस और अन्य सेलुलर प्रक्रियाओं को विनियमित करने के लिए जाना जाता है, एएलएस पैथोलॉजी में इसकी भूमिका स्पष्ट नहीं है। हाल ही में, भारतीय एएलएस रोगियों का आनुवंशिक विश्लेषण करते समय, हमारे समूह ने ओपीटीएन जीन में एक नए उत्परिवर्तन K489E की पहचान की। इसके अतिरिक्त, 25 भारतीय एएलएस रोगियों के रक्त डीएनए के संपूर्ण एक्सोम अनुक्रमण के माध्यम से, हमें सात भारतीय एएलएस रोगियों में समान उत्परिवर्तन मिला। यह एडेनिन से गुआनोसिन मिसेन्स उत्परिवर्तन ओपीटीएन के यूबिकिटिन-बाइंडिंग डोमेन में स्थित है। इस उत्परिवर्तन से जुड़े आणविक तंत्र की पहचान करने के लिए, हमने K489E OPTN उत्परिवर्तन वाले SH-SY5Y कोशिकाओं में एक इन-विट्रो सेल कल्चर-आधारित मॉडल विकसित किया। हमने देखा कि जंगली प्रकार की तुलना में K489E उत्परिवर्ती कोशिकाएं 36.5% अधिक मरती हैं। OPTN-K489E कोशिकाओं में मृत्यु के कारण का पता लगाने के लिए, हमने एपोप्टोसिस, नेक्रोप्टोसिस और ऑटोफैगी-मध्यस्थता वाले जीन और प्रोटीन की अभिव्यक्तियों को मापा। हमने miR-9, REST, CoREST और BDNF की जीन अभिव्यक्तियों में परिवर्तन और miRNA-9-मध्यस्थ एपोप्टोसिस के नियमन पर उनके ठोस प्रभाव को देखा। हमने जंगली प्रकार की तुलना में नेक्रोप्टोसिस-मध्यस्थता जीन RIPK1, RIPK3 और MLKL की अभिव्यक्तियों में भी परिवर्तन देखा। हमारे परिणाम ऑटोफैगी मध्यस्थ प्रोटीन TBK1, P62, और LC3II की अभिव्यक्तियों के अपग्रेडेशन को दर्शाते हैं। हमने इस उत्परिवर्तन का प्रभाव इम्यूनोप्रेसिपिटेशन, कन्फोकल माइक्रोस्कोपी और आरटी-पीसीआर के माध्यम से टीबीके1 जैसे इसके बाध्यकारी भागीदारों पर भी देखा है। वर्तमान परिदृश्य में, इस बीमारी का कोई स्थायी इलाज नहीं है, लेकिन शोधकर्ता विभिन्न दवाओं और विटामिनों का अध्ययन करके इस बीमारी को बढ़ने से रोकने के लिए लगातार प्रयास कर रहे हैं। इसी प्रकार, हमने SH-SY5Y के उत्परिवर्ती मॉडल पर विटामिन

सी और विटामिन डी के प्रभाव का भी अध्ययन किया है। हमने विटामिन सी के शामिल होने के बाद उत्परिवर्ती के मामले में कोशिका अस्तित्व में वृद्धि देखी है। हमने इस बात पर भी ध्यान केंद्रित किया है कि OPTN-K489E-व्यक्त करने वाली कोशिकाओं में यह कोशिका अस्तित्व कैसे हो रहा है।

TABLE OF CONTENTS

HIGHLIGHTS

The main findings of the study are as follows:

- Whole exome sequencing analysis shows remarkable variations in genome of Indian ALS patients than the healthy control
- Occurrence of reported mutation OPTN-K489E in Indian ALS patients
- OPTN-K489E variant of optineurin is pathogenic
- OPTN-K489E increases miR9, REST-CoREST and BDNF-mediated apoptosis
- OPTN-K489E upregulates RIPK1, RIPK3 and p-MLKL and causes necroptosis
- OPTN-K489E variant perturbs autophagy regulation
- FACS analysis reveals that apoptotic and necroptotic processes causes death in OPTN-K489E expressing cells
- Vitamin-C addition in SH-SY5Y cell expressing OPTN-K489E upregulates BDNF
- Vitamin-C supplementation causes upregulation of NRF2 iOPTN-K489E-expressingng cells
- Co-immunoprecipitation of optineurin and NRF2 shows more binding in presence of Vitamin-C
- Vitamin-C rescues cells from their death induced by OPTN-K489E

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

AKT	Protein kinase B
ALS	Amyotrophic lateral sclerosis
ANG	Angiogenin
ATXN2	Ataxin 2
BSA	Bovine serum albumin
BDNF	Brain derived neurotrophic factor
C9orf72	Chromosome 9 open reading frame 72
CCNF	Cyclin F
cDNA	Complementary DNA
CDSCO	Central Drugs Standard Control Organizations
CHCHD10	Coiled-coil-helix-coiled-coil-helix domain-containing protein 10
CHMP2B	Charged multivesicular body protein 2B
CoREST	Corepressor for element-1-silencing transcription factor
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***
EGFP	Enhanced green fluorescent protein
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
FIG4	Factor-induced gene 4
FTD	Frontotemporal dementia
FUS	Fused in sarcoma
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GFP	Green fluorescent protein
Glu/E	Glutamate
HCL	Hydrochloride
HNRNP	Heterogeneous nuclear ribonucleoprotein
HRP	Horseradish peroxidase
HSP	Hereditary Spastic Paraplegia
ICH	International Council for Harmonization
kDA	Kilo Dalton
KEAP1	Kelch-like ECH-associated protein 1
LC3-II	Light chain -II
LMN	Lower motor neurons
MATR3	Matrin 3
mRNA	Messenger RNA
miR9	Micro RNA-9
MLKL	Mixed lineage kinase doain-like pseudokinase

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
NRF2	Nuclear factor erythroid 2-related factor 2
OPMD	Oculopharyngeal muscular dystrophy
OPTN	Optineurin
P62	Sequestosome1
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
PDB	Protein Data Bank
PFN1	Profilin 1
PMSF	Phenyl methyl sulfonyl fluoride
PolyPhen	polymorphism phenotyping
PVDF	Polyvinylidene fluoride
REST	RE-1-silencing transcription factor
RIPA	Radioimmunoprecipitation
RIPK1	Receptor-interacting serine threonine protein kinase-1
RIPK3	Receptor-interacting serine threonine protein kinase-3
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
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
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