ELUCIDATION OF THE ROLE OF NEURONAL DIFFERENTIATION FACTORS BELONGING TO THE NEUROD FAMILY IN GLIOBLASTOMA

Anirban Jana

Department of Biochemical Engineering and Biotechnology

Indian Institute of Technology Delhi

May 2024

Elucidation of the Role of Neuronal Differentiation Factors belonging to the NeuroD family in Glioblastoma

by

Anirban Jana

Biochemical Engineering and Biotechnology

Submitted

in fulfilment of the requirements of the degree of

Doctor of Philosophy

to the

Indian Institute of Technology Delhi

May 2024

This thesis is dedicated to the most

important women in my life

my mother

Mrs. Sarbani Jana

and

my wife

Mrs. Kumarika Jana

and

my lovely daughter

Anika Jana

Certificate

This is to certify that the thesis entitled **"Elucidation of the Role of Neuronal Differentiation Factors belonging to the NeuroD family in Glioblastoma"** being submitted by **Mr. Anirban Jana** to the Indian Institute of Technology Delhi, for the award of the degree of **Doctor of Philosophy** is a record of bonafide research work carried out by him, which has been prepared under my supervision and guidance of conformity with the rules and regulations of Indian Institute of Technology Delhi. The research and the results presented in this thesis have not been submitted in part or full to any other University/ Institute for awarding any degree or diploma.

Prof Ritu Kulshreshtha

Professor

Department of Biochemical Engineering and Biotechnology

Indian Institute of Technology

New Delhi-110016

Date –

Place: New Delhi

Acknowledgments

Throughout my doctoral studies, I had help and support from many people, without whom my work would not have been possible. They have my sincere gratitude and privilege.

First and foremost, I want to express my gratitude to Prof. Ritu Kulshreshtha, who has been a tremendous help throughout the last five years. I am grateful to her for allowing me to work in her lab and pursue my Ph.D. in the Biochemical Engineering and Biotechnology department at IIT Delhi. She always encouraged me to attain my best potential and has helped me develop the analytical abilities needed for research. Working with her was encouraging, and the experience was incredible. Most importantly, my thesis would not have progressed to its current state without her guidance. Her recommendations, criticisms, and remarks aided me in expanding my knowledge of cancer biology.

I am grateful to Prof D. Sundar, Prof Preeti Srivastava, and Prof Vivekanandan Perumal (external expert from Kusuma School of Biological Sciences, IIT Delhi), members of my student research committee for monitoring my study progress and offering valuable insights and constructive recommendations. I also want to thank them for providing me access to their instrumentation facilities.

Prof. Chitra Sarkar, Dr. Vaishali Suri, and Dr. Vikas Sharma of the All India Institute of Medical Sciences (AIIMS, New Delhi) deserve special thanks for their assistance and productive collaboration. Their assistance and helpful advice were quite beneficial for me. Their aid and wise counsel were precious to me.

I would also like to thank Prof. Archana Chugh and Pankhuri Narula (Kusuma School of Biological Sciences, IIT Delhi) for including me as a collaborator that broadened my research aptitude.

I am very thankful to Dr. Kunzang and Dr. Khusboo for their support in performing some experiments and data acquisition that helped me properly shape my study.

I would like to express my gratitude to the Ministry of Education, Government of India, for their financial assistance during my study.

I want to express my gratitude to all who have ever been a part of the CBL lab, IIT Delhi, including Dr. Himesh, Dr. Rahul, Dr. Shivani, Dr. Sonam, Dr. Omkar, Dr. Srishti, Indranil,

Garima, Rahul, Aastha, Anuj, Anubha, Nidhi, Anuja, Rajat, Ravi, Ritanksha, and others for their support and valuable discussion. Sonam, Omkar, Srishti, Indranil, Garima, Anubha, Nidhi, Anuja, Rajat, and Himesh deserve appreciation for their unwavering love, support, and patience, as well as for providing me with some of my fondest Ph.D. memories.

My thanks are dedicated to my friends, especially Dr. Amit Kumar (from CSIR-Institute of Genomics and Integrative Biology, New Delhi), Dr. Surabhi Goyal, Dr. Deepak, Aditi Keshav, Adarsh, Nitika, Pravina, Dr. Sahil, Dr. Biju Jacobs, Swati (IIT Delhi) who helped me in arranging required chemicals, gave me access to the instruments and provided me support in my study throughout.

I appreciate the assistance of department staff members Anees, Sanjay, Ashish, Arjun, Yogesh, Sakshi, and Nitin. Their timely help and support helped me a lot.

Words fail me when it comes to expressing my thanks for my parent's support and love. I owe an immense debt of gratitude to my parents specially, my mother for their unwavering support while I was low, which helped me overcome all my obstacles and finish my thesis. I am also very thankful to my father-in-law, mother-in-law, and brother-in-law for their love and support. Without them, the situation could be more difficult for me. Dr. Ashirbad, my elder brother, deserves special praise for his affection and concern. I feel fortunate to have such a fantastic brother. I also like to thank my sister-in-law Joyashree Jana and my sweet niece Aashree for the unconditional love and support that made my journey easier.

Most importantly, I would like to thank my beautiful wife, Kumarika, and my daughter Anika for their support and motivation during rough times. I consider myself fortunate to have them in my life. They have been a massive inspiration and support for me throughout my journey. I consider myself lucky to be blessed with such a wonderfully supportive family.

Anirban Jana

.

Abstract:

Neuronal differentiation factors are a class of bHLH transcription factors recently shown to be differentially expressed in various cancers. However, their functions in glioblastoma (GBM) remain little studied so far. Previous studies from our group showed significant downregulation of neuronal differentiation factors, NeuroD2 and NeuroD6, in the TCGA-GBM patient cohort and further demonstrated the tumor-suppressive role of NeuroD2 in GBM under hypoxic conditions. However, the regulation or relevance of NeuroD6 downregulation remained unstudied so far. Here, we show that NeuroD6 is significantly downregulated in Indian GBM patients and GBM cell lines compared to the control brain. NeuroD6 and NeuroD2 both were found to be primarily enriched in the nuclear fraction as compared to the cytoplasmic fraction. Notably, analyses of the GBM patient dataset (TCGA, Firehose Legacy) using c-Bioportal, showed a truncating mutation in only one out of 619 patients, suggesting that NeuroD6 downregulation is not at the genomic level and may be regulated at the epigenetic, transcriptional or post-transcriptional levels. NeuroD6 was also transcriptionally induced by p53 and regulated at the post-transcriptional level by an oncogenic microRNA cluster, miR-421/374b. Our preliminary studies show that NeuroD6 may be regulated by DNA methylation. We further show that NeuroD6 functions as a tumor suppressor in GBM by inhibiting cell proliferation, migration, and tumor formation ability and promoting cell cycle arrest at the G_0G_1 phase in GBM cell lines T98G and A172. We also show that cells overexpressing NeuroD6 display increased apoptosis. Further studies revealed that both NeuroD2 and NeuroD6 regulate GBM cell metabolism by lowering glucose transport and lactate generation, indicating a reduction in glycolysis. Interestingly, overexpression of NeuroD6 and NeuroD2 was shown to lower the levels of oncogenic ID family molecules -ID1, ID2, and ID3 and has a negative feedback loop with ID2. In glucose-depleted conditions, both NeuroD2 and NeuroD6 were shown to accelerate GBM cell death, and when combined with siID2, the inhibition of proliferation was considerably increased. NeuroD2 and NeuroD6 transcript levels were found to be positively correlated in GBM tumors. Co-overexpression of NeuroD6 and NeuroD2 was shown to have a much stronger tumor-suppressive effect on cell proliferation, migration, and spheroid formation ability in GBM. Overall, our work reveals that NeuroD6 and NeuroD2, individually and in combination, may function as diagnostic biomarkers for GBM, and their overexpression may be an attractive option for GBM treatment.

सार:

न्यूरोनल विभेदन कारक बीएचएलएच प्रतिलेखन कारकों का एक वर्ग है जिसे हाल ही में विभिन्न कैंसर मेअलग-अलग रूप से व्यक्त किया गया है। हालाँकि, ग्लियोब्लास्टोमा (जीबीएम) में उनके कार्यों का अब तक बहुत कम अध्ययन किया गया है। हमारे समूह के पिछले अध्ययनों ने टीसीजीए-जीबीएम रोगी समूह में न्यूरोनल विभेदन कारकों, न्यूरोडी2 और न्यूरोडी6 में महत्वपूर्ण गिरावट देखी है और हाइपोक्सिक स्थितियों के तहत जीबीएम में न्यूरोडी2 की ट्यूमर-दमनकारी भूमिका का प्रदर्शन किया है। हालाँकि, न्यूरोडी6 डाउनरेगुलेशन के विनियमन या प्रासंगिकता का अब तक अध्ययन नहीं किया गया है। यहां, हम दिखाते हैं कि नियंत्रण मस्तिष्क की तुलना में भारतीय जीबीएम रोगियों और जीबीएम सेल लाइनों में न्यूरोडी6 को काफी कम विनियमित किया गया है। न्यूरोडी6 और न्यूरोडी2 दोनों को साइटोप्लाज्मिक अंश की तुलना में मुख्य रूप से परमाणु अंश में समृद्ध पाया गया। सी-बायोपोर्टल का उपयोग करके विश्लेषण किए गए पांच चार डेटासेट में से, केवल एक डेटासेट, ग्लियोब्लास्टोमा मल्टीफॉर्म (टीसीजीए, फायरहोज लिगेसी) डेटासेट ने केवल एक रोगी (0.17%) में एक ट्रंकिंग उत्परिवर्तन दिखाया, जो सुझाव देता है कि न्यूरोडी 6 डाउनरेगुलेशन जीनोमिक स्तर पर नहीं है और एपिजेनेटिक, ट्रांसक्रिप्शनल या पोस्ट-ट्रांसक्रिप्शनल स्तरों पर विनियमित किया जा सकता है। न्यूरोडी6 को भी पी53 द्वारा ट्रांसक्रिप्शनल रूप से प्रेरित किया गया था और पोस्ट-ट्रांसक्रिप्शनल स्तर पर एक ऑन्कोजेनिक माइक्रोआरएनए क्लस्टर, एमआईआर-421/374बी द्वारा विनियमित किया गया था। हमारे प्रारंभिक अध्ययन से पता चलता है कि न्यूरोडी6 को डीएनए मिथाइलेशन द्वारा नियंत्रित किया जा सकता है। हम आगे दिखाते हैं कि न्यूरोडी6 जीबीएम में ट्यूमर दमनकर्ता के रूप में कार्य करता है, जो कोशिका प्रसार, प्रवासन और ट्यूमर निर्माण क्षमता को रोकता है और जीबीएम सेल लाइनों टी98जी और ए172 में जी0जी1 चरण में कोशिका चक्र गिरफ्तारी को बढ़ावा देता है। हम यह भी दिखाते हैं कि न्यूरोडी6 को अधिक व्यक्त करने वाली कोशिकाएं एपोप्टोसिस को बढ़ाती हैं। आगे के अध्ययनों से पता चला कि न्यूरोडी2 और न्यूरोडी6 दोनों ग्लूकोज परिवहन और लैक्टेट उत्पादन को कम करके जीबीएम सेल चयापचय को नियंत्रित करते हैं, जो ग्लाइकोलाइसिस में कमी का संकेत देता है। दिलचस्प बात यह है कि न्यूरोडी6 और न्यूरोडी2 की अधिक अभिव्यक्ति से ऑन्कोजेनिक आईडी परिवार अणुओं -आईडी1, आईडी2 और आईडी3 के स्तर में कमी देखी गई है और आईडी2 के साथ एक नकारात्मक फीडबैक लूप है। ग्लूकोज-क्षीण स्थितियों में, न्यूरोडी2 और न्यूरोडी6 दोनों को जीबीएम कोशिका मृत्यु में तेजी लाने के लिए दिखाया गया था, और जब एसआईडी2 के साथ जोड़ा गया, तो प्रसार का अवरोध काफी बढ़ गया था। जीबीएम ट्यूमर में न्यूरोडी2 और न्यूरोडी6 ट्रांस्क्रिप्ट स्तर सकारात्मक रूप से सहसंबद्ध पाए गए। न्यूरोडी6 और न्यूरोडी2 के सह-

ओवरएक्प्रेशन को जीबीएम में कोशिका प्रसार, प्रवासन और गोलाकार गठन क्षमता पर अधिक मजबूत ट्यूमर-दमनकारी प्रभाव दिखाया गया है। कुल मिलाकर, हमारे काम से पता चलता है कि न्यूरोडी6 और न्यूरोडी2 व्यक्तिगत रूप से और संयोजन में जीबीएम के लिए डायग्नोस्टिक बायोमार्कर के रूप में कार्य कर सकते हैं, और उनकी ओवरएक्सप्रेशन जीबीएम उपचार के लिए एक आकर्षक विकल्प हो सकती है।

Table of Contents

List of Figures

List of Tables

Abbreviations

2D	Two Dimensional
3D	Three Dimensional
aa	Amino Acid
7AAD	7-Aminoactinomycin D
ABCG2	Atp Binding Cassette Subfamily G Member 2
Akt	Protein Kinase B
ALDOA	Aldolase A, Fructose-Bisphosphate
ALK	ALK Receptor Tyrosine Kinase
ALL	Acute Lymphocytic Leukemia
AMPK	AMP-Activated Protein Kinase
ANP32A	Acidic Nuclear Phosphoprotein 32 Family Member A
Apaf-1	Apoptotic Peptidase Activating Factor 1
ASCL1	Achaete-Scute Family Bhlh Transcription Factor 1
ATOH1	Atonal Bhlh Transcription Factor 1
ATP	Adenosine 5'-Triphosphate
ATRX	Atrx Chromatin Remodeler
BAX	Bcl2 Associated X, Apoptosis Regulator
BCL2	Bcl2 Apoptosis Regulator
BCL-xL	Bcl2 Like 1
BHF1	Neuronal Differentiation Factor
bHLH	Basic-Helix Loop Helix
Bim	BCL2 Like 11
BNCT	Boron Neutron Capture Therapy
BRAF	B-Raf Proto-Oncogene, Serine/Threonine Kinase
BRN2	Pou Class 3 Homeobox 2
BSA	Bovine Serum Albumin
CA9	Carbonic Anhydrase 9
CCND1	Cyclin D1
CCND2	Cyclin D2
CD133	Cluster Of Differentiation 133

CD24	Cd24 Antigen
CDK4	Cyclin Dependent Kinase 4
CDK6	Cyclin Dependent Kinase 6
cDNA	Complementary DNA
CGGA	Chinese Glioma Genome Atlas
ChIP-qPCR	Chromatin Immunoprecipitation-Quantitative Real Time PCR
CNS	Central Nervous system
CRC	Cyclic Redundancy Check
DCF	Dichloroflurescein
DCFDA	Dichloro Fluorescein Diacetate
DGCR8	Digeorge Syndrome Critical Region Gene 8
dHAND	Deciduum, Heart, Autonomic Nervous System And Neural Crest Derivatives-Expressed Protein 2
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic Acid
E2F	Retinoblastoma-Associated Protein 1
ECL	Enhanced Chemiluminescence
EGFR	Epidermal Growth Factor Receptor
eHAND	Extraembryonic Tissues, Heart, Autonomic Nervous System And Neural Crest Derivatives-Expressed Protein 1
EMT	Epithelial-Mesenchymal Transition
ENO1	Enolase 1
ENO2	Enolase 2
FABP7	Fatty Acid Binding Protein 7
FACS	Fluorescence Activated Cell Sorting
FBS	Fetal Bovine Serum
FCCP	Carbonyl Cyanide-P-Trifluoromethoxyphenylhydrazone
G6PD	Glucose-6-Phosphate Dehydrogenase
GABRA1	Gamma-Aminobutyric Acid Type A Receptor Subunit Alpha1
GAP43	Growth Associated Protein 43

GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
GBM	Gliobalstoma Multiforme
GLUT1	Glucose Transporter 1
GLUT2	Glucose Transporter 2
GLUT3	Glucose Transporter 3
GLUT4	Glucose Transporter 4
GSCs	Gbm Stem Cells
GTR	Guided Tissue Regeneration
HES	Hairy And Enhancer Of Split
HEY1	Hes Related Family Bhlh Transcription Factor With Yrpw Motif 1
HIF	Hypoxia Inducible Factor
HIF3A	Hypoxia Inducible Factor 3 Subunit Alpha
HK2	Hexokinase-2
HLH	Helix- Loop-Helix
HOXD10	Homeobox Protein Hox-D10
IC50	Half Maximal Inhibitory Concentration
ID1	Inhibitors Of Differentiation 1
ID2	Inhibitors Of Differentiation 2
ID3	Inhibitors Of Differentiation 3
ID4	Inhibitors Of Differentiation 4
IDH	Isocitrate Dehydrogenase 1
IGF	Insulin-Like Growth Factor
IgG	Immunoglobin G
IMRT	Intensity Modulated Radiotherapy
Kb	Kilobyte
LDH	Lactate Dehydrogenase
LDLR	Low Density Lipoprotein Receptor
LncRNAs	Long Noncoding Rnas
LRRFIP1	Leucine-Rich Repeat Flightless-Interacting Protein 1

Mash1	Mammalian Achaete Scute Homolog-1
MBD3	Methyl-Cpg-Binding Domain Protein 3
MDM2	Mouse Double Minute 2 Homolog
mETC	Mitochondrial Electron Transport Chain
miRISCs	Mirna-Induced Silencing Complexes
MMP2	Melanoma Mutants, Found Matrix Metalloproteinase-2
MMP9	Melanoma Mutants, Found Matrix Metalloproteinase-9
MRI	Magnetic Resonance Imaging
MSI1	Musashi RNA Binding Protein 1
MTDH	Metadherin
MTT	3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide
MYC	Myc Proto-Oncogene
MYF3	Myogenic Factor 3
MYF5	Myogenic Factor 5
MYOD	Myogenic Differentiation
MYOG	Myogenin
NAC	N-Acetyl Cysteine
NADH	Nicotinamide Adenine Dinucleotide
NEFL	Neurofilament Light Chain
NES	Nestin
NEUROD1	Neuronal Differentiation Factor 1
NEUROD2	Neuronal Differentiation Factor 2
NEUROD4	Neuronal Differentiation Factor 4
NEUROD6	Neuronal Differentiation Factor 6
NEUROG	Neurogenin
NEUROG2	Neurogenin 2
NF1	Neurofibromin 1
NFKB	Nuclear Factor Kappa B Subunit 1
NOS	Nitric Oxide Synthase
OCR	Oxygen Consumption Rate
OLIG2	Oligodendrocyte Transcription Factor 2
P16INK4	Cyclin Dependent Kinase Inhibitor 2a

P21	Cyclin Dependent Kinase Inhibitor 1a
TP53	Tumor Protein P53
PARP	Poly (Adp-Ribose) Polymerase
PBS	Phosphate-Buffered Saline
PDGF	Platelet-Derived Growth Factors
PDGFRA	Platelet-Derived Growth Factor Receptor Alpha
PDH	Pyruvate Dehydrogenase Complex
PDK1	Pyruvate Dehydrogenase Kinase 1
PDP	Pyruvate Dehydrogenase Phosphatase
PEP	Phosphoenolpyruvate
PGAM1	Phosphoglycerate Mutase 1
PI3K	Phosphoinositide 3-Kinase
PK	Pyruvate Kinase
PKLR	Pyruvate Kinase L/R
PKM	Pyruvate Kinase Muscle Isozyme
PKM2	Pyruvate Kinase Muscle Isozyme 2
pRB	Retinoblastoma-Associated Protein
PTEN	Phosphatase And Tensin Homolog
PUMA	P53 Upregulated Modulator Of Apoptosis
qPCR	Quantitative Polymerase Chain Reaction
Raf-1	Raf-1 Proto-Oncogene,
REST	Re1 Silencing Transcription Factor
RhoA	Ras Homolog Family Member A
RIPA	Radioimmunoprecipitation Assay Buffer
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
SDS	Sodium Dodecyl Sulfate
si-ID2	Id2 Silencing
SLC	Solute Carrier
SLC12A5	Solute Carrier Family 12 Member 5
SLIT2	Slit Guidance Ligand 2

SMAD4	Smad Family Member 4
SMARCA4	Swi/Snf Related, Matrix Associated, Actin Dependent Regulator Of Chromatin, Subfamily A, Member 4
SNAI2	Snail Family Transcriptional Repressor 2
SNAIL	Snail Family Transcriptional Repressor 1
SPRY2	Sprouty Rtk Signaling Antagonist 2
SREBP1	Sterol Regulatory Element Binding Transcription Factor 1
SYT1	Synaptotagmin 1
TBX3	T-Box Transcription Factor 3
TCA	Tricarboxylic Acid Cycle
TCGA	The Cancer Genome Atlas
TGF	Transforming Growth Factor
TGF2	Transforming Growth Factor 2
TMZ	Temozolomide
TNF	Tumor Necrosis Factor
TTFA	Thenoyltrifluoroacetone
TWIST	Twist Family Bhlh Transcription Factor 1
VEGF	Vascular Endothelial Growth Factor
WEE1	Wee1 G2 Checkpoint Kinase
WHO	World Health Organization
ZEB	Zinc Finger E-Box Binding Homeobox
ZEB1	Zinc Finger E-Box Binding Homeobox 1
ZEB2	Zinc Finger E-Box Binding Homeobox 2