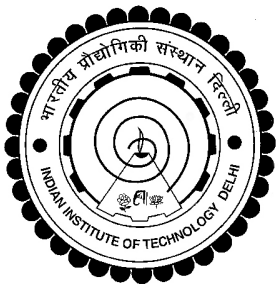
MOLECULAR MECHANISM OF HYPOXIA RESISTANCE IN GLIOBLASTOMA: ROLE OF MICRORNAS

RAHUL AGRAWAL



DEPARTMENT OF BIOCHEMICAL ENGINEERING AND BIOTECHNOLOGY

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MOLECULAR MECHANISM OF HYPOXIA RESISTANCE IN GLIOBLASTOMA: ROLE OF MICRORNAS

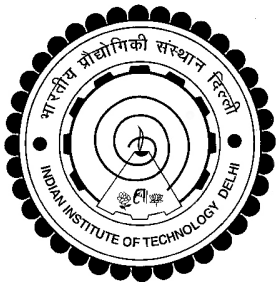
by

RAHUL AGRAWAL

Submitted

in fulfillment of the requirement of the degree of Doctor Of Philosophy

to the



Indian Institute of Technology Delhi

August 2017

Dedicated to
my loved ones

CERTIFICATE

This is to certify that the thesis entitled "**Molecular Mechanism of Hypoxia Resistance in Glioblastoma: Role of MicroRNAs**", being submitted by **Mr. Rahul Agrawal** to the Indian Institute of Technology Delhi, for the award of degree of **Doctor of Philosophy**, is a record of bonafide research work carried out by him, which has been prepared under our supervision and guidance of conformity with the rules and regulations of "Indian Institute of Technology Delhi". The research reports and the results presented in this thesis have not been submitted in part or full to any other University/ Institute for the award of any degree or diploma.

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ABSTRACT

Hypoxia is a critical aspect of the glioma microenvironment and has been associated with poor prognosis and resistance to various therapies. However, the mechanisms responsible for hypoxic survival of glioma cells remain unclear. Recent studies strongly suggest that microRNAs act as critical mediators of the hypoxic response. We thus hypothesized their prominent role in hypoxia resistance in glioblastoma (GBM) and aimed to identify those.

Here, we present the first detailed analysis of small RNA transcriptome of cell line U87MG, a grade IV glioma cell line, and its alteration under hypoxic condition. Based on deep sequencing and microarray data, we identify a set of hypoxia regulated microRNAs, a subset of which was validated in GBM cell lines U87MG and U251MG by stem-loop qRT-PCR. We show miR-210, miR-1275, miR-376c-3p, miR-23b-3p, miR-193a-3p and miR-145-5p to be up-regulated, while miR-92b-3p, miR-20a-5p, miR-10b-5p, miR-181a-2-3p and miR-185-5p are down-regulated by hypoxia. Notably, miR-210 and its isomiRs showed highest induction under hypoxia. Interestingly, certain hypoxia-induced miRNAs are also known to be over-expressed in GBM tumors, suggesting that hypoxia may be one of the factors involved in establishing the miRNA signature of GBM. Transcription factor binding sites for Hypoxia inducible factor 1 A (HIF1A) were identified in the promoter region (5 kb upstream) of 30 hypoxia-induced miRNAs. HIF-1A over-expression and silencing studies show regulation of specific miRNAs, including miR-210, to be HIF1A dependent. On the other hand, miR-210 leads to an increase in transcriptional activity of HIF and its target genes vascular endothelial growth factor (VEGF) and carbonic anhydrase 9 (CA9). MiR-210 levels were found to be high in astrocytic patient samples in a grade dependent manner and showed good correlation with the known hypoxia markers CA9 and VEGF. We show that miR-210 promotes cell proliferation, migration, hypoxic survival,

chemoresistance and inhibits apoptosis in GBM cells and targets a negative regulator of hypoxic response, HIF3A and a neuronal differentiation factor, NeuroD2. Our analyses of the TCGA-GBM data revealed significant downregulation of NeuroD2 in GBM patients. Low levels of NeuroD2 were found to be correlated with poor overall survival of GBM patients. NeuroD2 was shown to be transcriptionally induced by p53. NeuroD2 overexpression diminished GBM aggressiveness by inhibiting cell proliferation, migration and promoting apoptosis under hypoxia. NeuroD2 overexpressing glioma cells failed to form 3D-tumor spheroids and displayed reduced migration in a 3D gelatin matrix. NeuroD2 gene-signature was enriched in pathways belonging to cytokine-cytokine receptor interaction, TNF-signaling, PI3K-AKT signaling, focal adhesion and ECM-receptor interaction. Moreover, miR-210 mediated above mentioned multiple hallmarks of cancer were partly dependent on the targeted downregulation of NeuroD2.

Additionally, a total of 139 novel miRNAs were discovered by the analysis of deep sequencing data. Five novel miRNAs were validated by qRT-PCR, and three of these (iithsa_40, iithsa_15 and iithsa_92) were found to be differentially expressed under hypoxia. The regulation and the functional role of these novel miRNAs under hypoxic conditions remains to be seen.

Overall, our study reveals a novel miRNA signature of hypoxia in GBM and suggests miR-210 to be an oncogenic player and a novel potential intrinsic marker of hypoxia in GBM. Furthermore, our study identifies a novel role of NeuroD2 as a tumor suppressor in GBM the levels of which are tightly regulated by p53 and miR-210. Overexpressing NeuroD2 and/or inhibiting miR-210 may potentially be a simple and efficient therapeutic strategy to inhibit the malignant phenotype of GBM cells.

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ABBREVIATIONS

2-OG	2-oxoglutarate
2D	Two Dimensional
3D	Three Dimensional
7-AAD	7-Aminoactinomycin D
ACTB	Beta Actin
AGO	Argonaute
ANG-2	Angiogenin-2
ARNT	Aryl Hydrocarbon Receptor Nuclear Translocator
BAX	Bcl-2-associated X protein
BCL	B-cell Lymphoma
BCNU	B-chloro-nitrosourea
bHLH	Basic helix-loop-helix
BMP2	Bone Morphogenetic Protein 2
BNIP-3	BCL2/adenovirus E1B 19 kDa protein-Interacting Protein 3
CA9	Carbonic Anhydrase 9
CD133	Cluster of Differentiation 133
cDNA	Complementary DNA
ChIP	Chromatin Immunoprecipitation
CNS	Central Nervous System
COL1A1	Collagen type 1 Alpha 1 chain
CSC	Cancer Stem Cells
CT	Computerized Tomography
CUL2	Cullin2
CXCL5	C-X-C motif chemokine 5
ECM	Extra Cellular Matrix
EGFR	Epidermal Growth Factor Receptor
EMT	Epithelial to Mesenchymal Transition
EtOH	Ethanol
FACS	Fluorescence-activated Cell Sorting
FAK	Focal Adhesion Kinase
FBS	Fetal Bovine Serum
GABRA1	Gamma-aminobutyric acid Receptor subunit Alpha-1
GAPDH	Glyceraldehyde 3-phosphate Dehydrogenase
GBM	Glioblastoma
GPD1L	Glycerol-3-phosphate dehydrogenase 1-like
GRB7	Growth factor Receptor Bound protein 7
H&E	Haematoxylin- and Eosin

HER2	Human Epidermal growth factor Receptor 2
HIF1A	Hypoxia Inducible Factor1A
HOXD10	Homeo box DNA binding domain 10
HRE	Hypoxia Response Element
HRM	Hypoxia-Regulated microRNAs
IAP-2	Inhibitor of Apoptosis 2
ID1	Inhibitor of DNA binding 1
IDH1	Isocitrate Dehydrogenase1
IHC	Immunohistochemistry
IKZF3	Ikaros family Zinc Finger protein 3
LIF	Leukemia Inhibitory Factor
lincRNA	Long Intervening Non Coding RNAs
MAP	Microtubule Associated Protein 2
MAPK	Mitogen Activated Protein Kinase
MAZ	Myc Associated Zinc finger protein
MDM2	Mouse Double Minute 2 homolog
MGMT	O-6-Methyl Guanine DNA Methyl Transferase
MMP	Matrix Metalloproteases
MNT	MAX Network Transcription repressor
MRI	Magnetic Resonance Imaging
mRNA	Messenger RNA
m-TOR	Mechanistic/mammalian Target of Rapamycin
MTT	3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide
NEFL	Neurofilament Light
NeuroD2	Neuronal Differentiation 2
NF1	Neurofibromin1
NFKBIA	NF-kappa-B-Inhibitor Alpha
NGS	Next Generation Sequencing
NOC	N-nitroso Compounds
Oct-4	Octamer-binding transcription factor 4
ODDD	Oxygen Dependent Degradation Domain
OPN	Osteopontin
PBS	Phosphate Buffer Saline
PDGFR	Platelet Derived Growth Factor Receptor
PHDs	Prolyl Hydroxylases
PI3K	Phosphoinositide 3-Kinase
piRNA	Piwi-interacting RNA
Poly-HEMA	Poly (2-hydroxyethyl methacrylate)
PPARγ	Peroxisome Proliferator-activated Receptors γ
PTP1b	Protein Tyrosine Phosphatase 1B

PUMA	p53 Upregulated Modulator of Apoptosis
qRT-PCR	Quantitative Reverse Transcription Polymerase Chain Reaction
RECK	Reversion Inducing Cysteine Rich Protein With Kazal Motifs
REST	Repressor Element 1 Silencing Transcription Factor
RHOA	Ras homolog gene family, member A
RISC	RNA-induced Silencing Complex
RPKM	Reads per Kilobase per Million
rRNA	Ribosomal RNA
scRNA	Small cytoplasmic RNA
SDHD	Succinate Dehydrogenase Complex Subunit D
SDS	Sodium Dodecyl Sulfate
SLC12A5	Solute carrier family 12 member 5
snoRNA	Small nucleolar RNA
SOX-2	(Sex determining region Y)-box-2
SYT1	Synaptogemin1
TAD	Transactivation Domain
TCGA	The Cancer Genome Atlas
TF	Transcription Factors
TGF	Transforming Growth Factor
TIMP3	Metalloproteinase Inhibitor 3
TMZ	Temozolomide
TNF	Tumor Necrosis Factor
TPM	Transcript Parts per Million
TRANSFAC	TRANScription FACtor database
tRNA	Transfer RNA
TSC	Trans Sodium Crocetinate
ULA	Ultra-low Attachment
PAGE	Poly-Acryl amide Gel Electrophoresis
UTR	Untranslated Region
VEGF	Vascular Endothelial Growth Factor
VHL	Von-Hippel-Lindau
ZEB1	Zinc finger E-box-binding homeobox 1