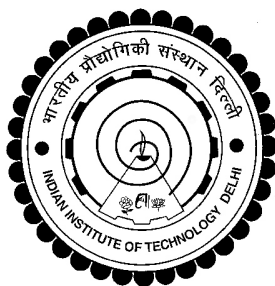


MOLECULAR MECHANISM OF HYPOXIA RESISTANCE IN GLIOBLASTOMA: ROLE OF MICRORNAS

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INDIAN INSTITUTE OF TECHNOLOGY DELHI

AUGUST 2017

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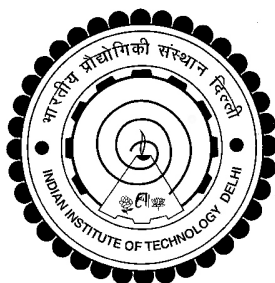
by

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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ACKNOWLEDGEMENTS

It is my great pleasure and privilege to thank everyone who has guided and helped me during my PhD work. Without their guidance and support, this dissertation would not be finished.

I would like to express my heartfelt gratitude to my research supervisor Dr. Ritu Kulshreshtha for offering me the opportunity to pursue my PhD in the Department of Biochemical Engineering and Biotechnology at IIT Delhi. Without her guidance, constant encouragement and ever inspiring support this thesis could not have reached its present form. Her comments, suggestions and criticism gave me considerable help to extend my understanding of cancer biology in order to successfully shape this work.

I am extremely thankful to Prof. P. K. Roychoudhury for his constant support and encouragement. I am grateful to him for allowing me to use his lab facilities. His enthusiasm guided me all the way during my Ph. D. tenure.

I am very much grateful to Prof. Chitra Sarkar, AIIMS for her guidance, support and critical evaluation which steered the work towards scientific excellence. All those fruitful discussions and comments received have helped me in improving my scientific foundations and channeled towards a prospective scientific career.

I wish to express my sincere thanks to my SRC members, Prof. Prashant Mishra, Dr. Preeti Srivastava and Dr. Vivekanandan Perumal (external expert, Kusuma School of Biological Sciences, IIT Delhi) for monitoring my work progress and giving valuable suggestions.

I am thankful to Vikas, Prit and Prerana, AIIMS for their help and support. Their support with desired experiments, clarification of doubts and valuable suggestions helped me a lot.

I am very thankful to all my friends, especially Ravi, Puneet, Vishal, Sudhaker, Nishant Sir and Ravi Sir who supported me all through my good and bad times and for giving me a helping hand during this whole process.

My thanks are dedicated to all the members in my group. Especially Shivani, Neha, Sonam, Omkar, Shikha, Srishti, Ankita, Sonali, Deependra, Vasu are highly acknowledged for the valuable discussions, conversations and nice atmosphere in the laboratory. I would also like to acknowledge many other colleagues in IIT, especially Neeti, Sanjay, Aman, Sumit, Swati, Shilpi and Ritesh for answering many of my questions.

I am thankful to my friend Khushboo for her ceaseless encouragement and cheerful banter to lift me up when I was down.

I am extremely thankful to Yogesh Bhaiya, Rana Sir and Sunil for their constant help and support. I wish to thank Kirti, Gauri, Neera Mam, Bhagwan Das ji, Ratan Sir, SitaRam ji and Mukesh Sir for their timely help and support.

I acknowledge Department of Biotechnology (DBT), New Delhi for providing the financial support during the thesis work.

Finally, I take this opportunity to express my deepest gratitude to my family who wholeheartedly supported me and expressed unconditional love without which my journey would have been a dream. I consider myself lucky for being blessed with such wonderful parents, who helped me realize my dreams. I have been most fortunate to have the untiring support and understanding of my brother and sisters who constantly encouraged my passion for science.

Most important of all, I would like to thank my beautiful wife Garima for her unconditional love and support throughout the journey of thesis submission. She has been a huge source of inspiration for me. She always motivated me and firmly stood beside me through all ups and downs. Her understanding and encouragement when the times got rough are much appreciated and duly noted.

Finally, in praise of the Almighty I remember him fondly for sheltering me in all aspects of life.

Rahul Agrawal

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.

CONTENTS

Title	Page No.
CERTIFICATE.....	i
ACKNOWLEDGEMENTS.....	ii
ABSTRACT.....	v
CONTENTS.....	vii
LIST OF FIGURES.....	xi
LIST OF TABLES.....	xiii
ABBREVIATIONS.....	xiv
Chapter 1 INTRODUCTION.....	1
Chapter 2 OBJECTIVES.....	6
Chapter 3 REVIEW OF LITERATURE.....	9
3.1 Glioblastoma.....	10
3.1.1 Signs and symptoms.....	10
3.1.2 Risk factors.....	11
3.1.3 Classification.....	11
3.1.4 Diagnosis and prognosis.....	13
3.1.5 Treatment.....	14
3.1.5.1 Symptomatic therapy.....	15
3.1.5.2 Palliative therapy.....	15
3.1.5.2a Surgery.....	15
3.1.5.2b Radiotherapy.....	15
3.1.5.2c Chemotherapy.....	16
3.2 Hypoxia.....	17

3.2.1 Hypoxia inducible factors.....	18
3.2.2 HIF regulating pathways.....	19
3.2.3 Cellular responses to hypoxia.....	21
3.2.3.1 Apoptosis.....	21
3.2.3.2 Cell proliferation.....	21
3.2.3.3 Differentiation.....	22
3.2.3.4 Migration.....	23
3.2.3.5 Angiogenesis.....	23
3.3 MiRNA.....	23
3.3.1 Biogenesis.....	24
3.3.2 Mode of action.....	26
3.3.3 Cellular functions.....	28
3.4 Glioblastoma and miRNAs.....	29
3.5 Hypoxia and miRNA.....	33
Chapter 4 MATERIALS AND METHODS.....	39
4.1 Cell culture.....	40
4.2 Astrocytoma patient samples.....	40
4.3 Transient transfections.....	41
4.4 Construction of stable polyclonal cell lines.....	41
4.5 Hypoxia, serum starvation and drug treatment.....	41
4.6 RNA isolation.....	42
4.7 Small RNA preparation.....	42
4.8 Analysis of deep sequencing data.....	42
4.9 Expression pattern of known miRNAs.....	43
4.10 Data normalization.....	43

4.11 Differentially expressed miRNAs.....	44
4.12 Identification of novel miRNAs.....	44
4.13 IsomiRs of novel miRs.....	44
4.14 Microarray expression profiling and analysis.....	45
4.15 MiRNA and mRNA quantitation.....	45
4.16 Target prediction of miR-210 and differentially expressed novel miRs.....	45
4.17 Prediction of HREs in the promoter of miRNAs.....	46
4.18 Construction of 3'-UTR-luciferase or promoter-luciferase constructs.....	46
4.19 Dual luciferase assay.....	46
4.20 Caspase-3/7 activity assay.....	47
4.21 PE Annexin V apoptosis detection assay.....	47
4.22 Generation of 3D tumor spheroids.....	47
4.23 Tumor spheroid-based migration assay.....	48
4.24 Chromatin Immunoprecipitation-qPCR (ChIP-qPCR).....	49
4.25 Western blot.....	49
4.26 Cell proliferation assay.....	50
4.27 Soft agar assay.....	50
4.28 Boyden chamber assay.....	50
4.29 Immunohistochemistry.....	51
4.30 Statistical analyses.....	51
Chapter 5 RESULTS.....	52
5.1 Identification of small RNA profiles associated with hypoxia in GBM.....	53
5.1.1 MicroRNA signature of hypoxia in GBM.....	53
5.1.2 Discovery of novel miRNAs.....	69
5.1.3 MiR-210 a putative novel hypoxia marker in GBM patients.....	71

5.2 Study HIF mediated induction of candidate miRNAs.....	74
5.3 Study functions of candidate miRNA in GBM.....	76
5.3.1 MiR-210 promotes cell proliferation, anchorage independence and migration in GBM	76
5.3.2 MiR-210 inhibits apoptosis in GBM.....	78
5.3.3 MiR-210 promotes hypoxia/stress/chemoresistance in GBM.....	79
5.3.4 miR-210 promotes HIF transcriptional activity.....	81
5.4 Identify target transcripts of candidate miRNA in GBM.....	82
5.4.1 HIF3A and NeuroD2 are direct targets of miR-210.....	82
5.4.2 Expression pattern of HIF3A in GBM.....	84
5.4.3 Expression pattern of miR-210 and NeuroD2 in different grades of astrocytoma	85
5.4.4 NeuroD2 functions as a tumor suppressor under hypoxia in GBM.....	90
5.4.5 NeuroD2 inhibits 3D tumor spheroid forming capacity of GBM cells.....	96
5.4.6 NeuroD2 gene signature under hypoxia in GBM.....	100
5.4.7 Identification of the role of p53/NeuroD2/miR-210 axis in regulation of hypoxic responses in GBM	104
5.4.8 NeuroD2 promotes apoptosis under hypoxia in GBM.....	107
Chapter 6 DISCUSSION.....	109
Chapter 7 CONCLUDING REMARKS AND FUTURE DIRECTIONS.....	118
7.1 Concluding remarks.....	119
7.2 Future directions.....	121
BIBLIOGRAPHY.....	124
APPENDIX.....	160
RESUME OF THE AUTHOR.....	186

LIST OF FIGURES

Figure No.	Title	Page No.
3.1	Clinically relevant subtypes of GBM as defined by Verhaak et al, 2010.	12
3.2	Domain structure of human HIF1 α and HIF1 β .	19
3.3	Pictorial representation of miRNA biogenesis.	25
3.4	Mode of action of miRNAs in plants and animals.	27
3.5	Pictorial representation of the impact of miRNAs over major signaling pathways affected in gliomagenesis.	30
5.1	Size distribution graph.	54
5.2	Expression level of small RNAs.	55
5.3	Expression of known miRNAs is represented in the form of frequency count v/s no. of miRNAs in normoxia and hypoxia samples.	56
5.4	The abundance of highly expressed [$>10,000$ counts] (a) and low expressed [<10 counts] (b) select miRNAs in normoxic and hypoxic cells.	57
5.5	MiRNA cluster analyses.	63
5.6	Figure showing location of HREs in the promoters of hypoxia-induced miRNAs, predicted by the program PROMO.	66
5.7	Quantitative RT-PCR data showing miRNAs that are upregulated (a, c) or downregulated (b, d) in response to hypoxia in U87MG and A172 cells, respectively.	68
5.8	Identification of novel miRNAs.	71
5.9	Quantitation and correlation of miR-210-3p levels with hypoxia markers VEGF and CA9.	73
5.10	Quantitative RT-PCR data showing miRNA levels in response to HIF1A.	75
5.11	MiR-210 promotes cell proliferation, anchorage independence and migration in U87MG and A172 cells.	77

5.12	MiR-210 promotes apoptosis in GBM cell lines.	78
5.13	MiR-210 promotes stress resistance in GBM cells.	80
5.14	MiR-210 induces HIF transcriptional activity.	81
5.15	MiR-210 targets HIF3A and NeuroD2.	84
5.16	Quantitation and correlation of levels of HIF3A with miR-210.	85
5.17	Expression pattern of miR-210 and NeuroD2 in astrocytoma patients.	89
5.18	MiR-210 mediated cell proliferation, anchorage independence and migration is dependent on targeted downregulation of NeuroD2 in hypoxic microenvironment.	95
5.19	NeuroD2 inhibits 3D tumor spheroids forming capacity of GBM cells and their migration.	100
5.20	Functional classification of NeuroD2 regulated genes under hypoxic condition.	103
5.21	NeuroD2 is regulated by p53 under hypoxic conditions in GBM.	106
5.22	NeuroD2 promotes apoptosis under hypoxia in GBM.	108
7.1	A schematic view of regulation of miR-210 and its targets.	120

LIST OF TABLES

Table No.	Title	Page No.
5.1	The known piRNA expression pattern in normoxia and hypoxia.	58
5.2	MiRNA cluster analysis of the known miRNAs in normoxic and hypoxic U87MG cells	62
5.3	A table showing correlation of microRNAs altered in hypoxia or in GBM tumor tissues	65

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)
PPAR γ	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-Acyl amide Gel Electrophoresis
UTR	Untranslated Region
VEGF	Vascular Endothelial Growth Factor
VHL	Von-Hippel-Lindau
ZEB1	Zinc finger E-box-binding homeobox 1