# UNRAVELLING THE ROLE OF MIR-196A IN GLIOBLASTOMA


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# UNRAVELLING THE ROLE OF MIR-196A IN GLIOBLASTOMA

**by**


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# CERTIFICATE

This is to certify that the thesis entitled "**Unravelling the role of miR-196a in glioblastoma**", being submitted by **Ms. Sonam Takkar** 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 her, which has been prepared under my 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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**Place:**

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# ABSTRACT

Glioblastoma (GBM) is the most common and aggressive malignant primary brain tumor in humans. Despite of advances in medical management of solid tumors, the mortality rates of GBM patients remain high, which urge for a better understanding of GBM pathogenesis and improvement in its therapeutic strategy. Hypoxia has been correlated with the aggressive form of glial tumors, their poor prognosis and resistance to various therapies. MicroRNAs (miRNAs) have emerged as key players in cellular transformation and tumorigenesis and have shown great potential for cancer diagnostics and therapeutics. The present study is based on previous studies from our lab showing alteration in the miRNA profile in GBM cell line U87MG in response to severe hypoxia. Here, we take forward our previous studies to identify hypoxia regulated miRNAs that play critical role in hypoxia signalling and studying its clinical implications. A total of sixteen miRNAs were further validated by qRT-PCR and nine were found to be hypoxia regulated in GBM cells. Among these, miR-196a was found to be highly induced in response to hypoxia in a HIF dependent manner. miR-196a was also found to be significantly up-regulated in TCGA-GBM and Indian GBM patient cohorts. The high expression of miR-196a was shown to be associated with poor prognosis in GBM patients. We did functional characterization of miR-196a in GBM cell lines, U87MG and A172 using both and inhibition and overexpression approach in normoxia as well as hypoxia. miR-196a overexpression was found to induce cellular proliferation, migration and colony forming potential and inhibit apoptosis in U87MG and A172 cell lines while miR-196a inhibition using anti-miR-196a showed opposite results suggesting oncogenic role of miR-196a in GBM. We further identified targets of miR-196a using a combination of bioinformatic and biochemical approaches. Notably, we found that miR-196a not only downregulates tumor suppressor genes but may also be involved in up-regulating the levels of specific oncogenes. We further unveiled that NRAS, AJAP1, TAOK1 and COL24A1 are direct targets of miR-196a. Our study also reported complex competitive regulation of oncogenic NRAS by miR-196a, miR-146a and let-7g in GBM. Analysis of microarray gene expression data obtained by miR-196a inhibition under hypoxia elucidated the role of miR-196a in HIF, Calcium Adhesion, Wnt and Cell Adhesion pathways. Interestingly, miR-196a was found to positively regulate the expression of various genes involved in induction or stabilization of HIFs and maintenance of hypoxic conditions, thereby suggesting the existence of an indirect miR-196a/HIF positive loop under hypoxia. Overall,

our work identifies novel axis of HIF-1/miR-196a/NRAS in GBM and suggests its prognostic and therapeutic significance.

# सार

ग्लियोब्लास्टोमा (जीबीएम) मनुष्यों में सबसे आम और आक्रामक घातक प्राथमिक ब्रेन ट्यूमर है। ठोस ट्यूमर के चिकित्सा प्रबंधन में प्रगति के बावजूद, जीबीएम रोगियों की मृत्यु दर उच्च बनी हुई है, जो जीबीएम रोगजनन की बेहतर समझ और इसकी चिकित्सीय रणनीति में सुधार के लिए आग्रह करते हैं। हाइपोक्सिया को ग्लायल ट्यूमर के आक्रामक रूप, उनके खराब रोग का निदान और विभिन्न उपचारों के प्रतिरोध के साथ सहसंबद्ध किया गया है। सूक्ष्म आरएनए (miRNAs) सेलुलर ट्रांसफॉर्मेशन और ट्यूमरजेनिसिस में प्रमुख खिलाड़ी के रूप में उभरे हैं और कैंसर निदान और उपचार के लिए काफी संभावनाएं दिखाई हैं। वर्तमान अध्ययन गंभीर हाइपोक्सिया के जवाब में जीबीएम सेल लाइन U87MG में miRNA प्रोफ़ाइल में परिवर्तन दिखाने वाली हमारी लैब से पिछले अध्ययनों पर आधारित है। यहाँ, हम हाइपोक्सिया विनियमित miRNAs की पहचान करने के लिए अपने पिछले अध्ययनों को आगे बढ़ाते हैं जो हाइपोक्सिया सिग्नलिंग में महत्वपूर्ण भूमिका निभाते हैं और इसके नैदानिक प्रभाव का अध्ययन करते हैं। कुल सोलह miRNAs को आगे क्यूआरटी-पीसीआर द्वारा मान्य किया गया था और नौ जीबीएम कोशिकाओं में नियंत्रित हाइपोक्सिया पाए गए थे। इनमें, एमआईआर-196ए (miR-196a) को एचआईएफ निर्भर तरीके से हाइपोक्सिया के जवाब में अत्यधिक प्रेरित पाया गया। miR-196a को टीसीजीए-जीबीएम और भारतीय जीबीएम रोगी सहकर्मियों में भी काफी हद तक नियंत्रित किया गया। miR-196a की उच्च अभिव्यक्ति को जीबीएम रोगियों में खराब रोगनिरोध से जुड़ा हुआ दिखाया गया था। हमने दोनों जीबीएम सेल लाइनों, U87MG और A172 मे miR-196a के कार्यात्मक लक्षण वर्णन और नॉरमोक्सिया के साथ-साथ हाइपोक्सिया में निषेध और ओवरएक्प्रेशन दृष्टिकोण का उपयोग किया। miR-196a अभिव्यक्ति में वृद्धि A172 और U87MG सेल लाइनों मे  मेंसेलुलर प्रसार, माइग्रेशन और कॉलोनी बनाने के लिए संभावित पाया गया था और एपोप्टोसिस को कम करने के लिए पाया गया था, जबकि miR-196a ने एंटी-miR-196a का उपयोग करते हुए विपरीत परिणाम दिखाए थे, जीबीएम में miR-196a की ऑन्कोजेनिक भूमिका का सुझाव दिया था। हमने आगे जैव-रासायनिक और जैव-रासायनिक दृष्टिकोणों के संयोजन का उपयोग करके miR-196a के लक्ष्यों की पहचान की। विशेष रूप से, हमने पाया कि miR-196a न केवल ट्यूमर शमन जीन को डाउनग्रेड करता है, बल्कि विशिष्ट ऑन्कोजेन्स के स्तर को विनियमित करने में भी शामिल हो सकता है। हमने आगे खुलासा किया कि NRAS, AJAP1, TAOK1 और COL24A1 miR-196a के प्रत्यक्ष लक्ष्य हैं। हमारे अध्ययन ने जीबीएम में miR-196a, miR-146a और let-7g द्वारा ऑन्कोजेनिक NRAS के जटिल प्रतिस्पर्धी विनियमन की भी सूचना दी। हाइपोक्सिया के तहत miR-196a निषेध द्वारा प्राप्त माइक्रोएरे जीन अभिव्यक्ति डेटा का विश्लेषण HIF, कैल्शियम आसंजन, डब्ल्यूएनटी और सेल आसंजन मार्गों में miR-196a की भूमिका को स्पष्ट करता है। दिलचस्प बात यह है कि miR-196a को HIF की प्रेरण या स्थिरीकरण और हाइपोक्सिक स्थितियों के रखरखाव में शामिल विभिन्न जीनों की अभिव्यक्ति को सकारात्मक रूप से विनियमित करने के लिए पाया गया था, जिससे हाइपोक्सिया के तहत एक अप्रत्यक्ष miR-196a / HIF पॉजिटिव लूप के अस्तित्व का सुझाव दिया गया था। कुल मिलाकर, हमारा काम जीबीएम में HIF-1 / miR-196a / NRAS के उपन्यास अक्ष की पहचान करता है और इसके पूर्वानुमान और चिकित्सीय महत्व का सुझाव देता है।

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# ABBREVIATIONS

| | |
|---|---|
| **AGO2** | Argonaute |
| **AJAP1** | Adherens Junctions Associated Protein 1 |
| **AKT** | Protein kinase B |
| **ARID4B** | AT-Rich Interaction Domain 4B |
| **ARNT** | Aryl Hydrocarbon Receptor Nuclear Translocator |
| **ATP** | Adenosine Triphosphate |
| **ATRX** | ATP-dependent helicase |
| **Bcl-2** | B-cell lymphoma 2) |
| **bHLH** | basic Helix-Loop-Helix |
| **BIRC6** | Baculoviral IAP repeat-containing protein 6 |
| **BSA** | Bovine Serum Albumin |
| **CA9** | Carbonic Anhydrase 9 |
| **CDK6** | Cyclin-dependent kinase 6 |
| **cDNA** | Complementary DNA |
| **$CO_2$** | Carbon dioxide |
| **COL3A1** | Collagen Type III Alpha 1 Chain |
| **COL24A1** | Collagen type XXIV alpha 1 chain |
| **CSC** | Cancer Stem Cells |
| **CT** | Computed Tomography |
| **COX2** | Cyclooxygenase-2 |
| **CREB** | cAMP response element-binding protein |
| **CYBB** | NADPH oxidase 2 |
| **DGCR8** | DiGeorge syndrome chromosomal [or critical] region 8 |
| **DNA** | Deoxyribonucleic acid |
| **ECL** | Enhanced chemiluminescence |
| **ECM** | Extracellular matrix |
| **EGFR** | Epidermal Growth Factor Receptor |
| **EMT** | Epithelial to Mesenchymal Transition |
| **EP300** | E1A Binding Protein P300 |
| **EPO** | Erythropoietin |
| **ERBB2** | Erythroblastic oncogene B |
| **FACS** | Fluorescence-activated Cell Sorting |
| **FBS** | Fetal Bovine Serum |
| **FIH** | Factor-inhibiting HIF |
| **FOXO1** | Forkhead Box O1 |
| **GABRA1** | Gamma-aminobutyric acid type A receptor alpha 1 subunit |
| **GAPDH** | Glyceraldehyde 3-Phosphate Dehydrogenase |
| **GATA1** | GATA Binding Protein 1 |
| **GBM** | Glioblastoma Multiforme |
| **GGT1** | Gamma-Glutamyltransferase 1 |
| **GLUTI** | Glucose transporter |
| **HDAC1** | Human histone deacetylase 4 |
| **H & E** | Haematoxylin- and Eosin |
| **HGI2** | Hypoxia-inducible protein 2 |
| **HIF** | Hypoxia Inducible Factor |
| **HIF-1α** | Hypoxia Inducible Factor1A |

| | |
|---|---|
| **HOX** | Homeobox |
| **HRE** | Hypoxia Response Element |
| **HRM** | Hypoxia Regulated microRNAs |
| **HRP** | Horseradish peroxidase |
| **IDH1** | Isocitrate Dehydrogenase*1* |
| **iNOS** | Inducible nitric oxide synthase |
| **KPS** | Karnofsky Performance Scale |
| **lincRNA** | Large intergenic noncoding RNAs |
| **MAPK** | Mitogen-Activate Protein Kinases |
| **MGMT** | O-6-Methyl Guanine DNA Methyl Transferase |
| **miRISCs** | miRNA-induced silencing complexes |
| **mRNA** | Messenger RNA |
| **miRNA/miR** | MicroRNA |
| **MMP** | Matrix Metalloproteases |
| **MREs** | miRNA response elements |
| **MRI** | Magnetic Resonance Imaging |
| **mTOR** | Mammalian target of rapamycin |
| **MTT** | 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide |
| **NCCS** | National Centre for Cell Sciences |
| **NEFL** | Neurofilament |
| **NF1** | Neurofibromin1 |
| **NFκB** | Nuclear Factor kappa B |
| **NFκBIA** | NF-kappa-B-Inhibitor Alpha |
| **NRAS** | NRAS Proto-Oncogene |
| **ncRNAs** | non-coding RNAs |
| **ODD** | Oxygen-Dependent Degradation domain |
| **OPN** | Osteopontin |
| **PARP** | poly-ADP ribose polymerase |
| **PAS** | Per-Arnt-Sim |
| **PBS** | Phosphate Buffer Saline |
| **PBST** | Phosphate Buffer Saline with Tween-20 |
| **PCR** | Polymeraase Chain Reaction |
| **PDGFRA** | Platelet Derived Growth Factor Receptor |
| **PE** | Phycoerythrin |
| **PET** | Positron emission tomography |
| **PET membrane** | Polyethylene Terephthalate membrane |
| **PHDs** | prolyl-hydroxylases |
| **PI3K** | phosphoinositide 3-kinase |
| **PIPES** | piperazine-N,N′-bis(2-ethanesulfonic acid) |
| **PMSF** | Phenylmethylsulfonyl fluoride |
| **PPARγ** | Peroxisome proliferator-activated receptor gamma |
| **pri-miRs** | primary-miRNAs |
| **PTEN** | Phosphatase and tensin homolog |
| **PUMA** | p53 up-regulated modulator of apoptosis |
| **qRT-PCR** | Quantitative Reverse Transcription-PCR |
| **qPCR** | Quantitative real-time PCR |
| **RECK** | Reversion-inducing-cysteine-rich protein with kazal motifs |
| **RhoA** | Ras homolog gene family, member A |

| | |
|---|---|
| **RNA** | Ribonucleic acid |
| **ROCK** | Rho-associated coiled-coil kinases |
| **ROS** | Reactive oxygen species |
| **RT** | Room Temperature |
| **RNA** | Ribonucleic Acid |
| **RUFY3** | RUN And FYVE Domain Containing 3 |
| **SDS-PAGE** | Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis |
| **shRNA** | short hairpin RNA |
| **SLC12A5** | Solute carrier family 12 member 5 |
| **sRNA-Seq** | Small RNA Sequencing(deep sequencing) |
| **STAT3** | Signal transducer and activator of transcription 3 |
| **STY1** | Synaptotgamin 1 |
| **TAOK1** | Thousand and One Amino Acid Protein Kinase 1 |
| **TARPB2** | TAR RNA-BINDING PROTEIN 2 |
| **TCEAL7** | Transcription Elongation Factor A Like 7 |
| **TCGA** | The Cancer Genome Atlas |
| **TEAD3** | TEA domain transcription factor 3 |
| **TGF-β** | Transforming growth factor beta |
| **TIMP3** | TIMP Metallopeptidase Inhibitor 3 |
| **TMZ** | Temozolomide |
| **TRANSFAC** | TRANScription FACtor database |
| **TWIST1** | Twist-related protein 1 |
| **TWIST2** | Twist Family BHLH Transcription Factor 2) |
| **UBE2C** | Ubiquitin-conjugating enzyme E2C |
| **UTR** | Untranslated Region |
| **VEGF** | Vascular Endothelial Growth Factor |
| **VHL** | Von Hippel- Lindau |
| **WHO** | World Health Organization |
| **XPO5** | Exportin-5 |
| **ZEB 1** | Zinc finger E-box-binding homeobox 1 |
| **ZEB 2** | Zinc finger E-box-binding homeobox 2 |
| **ZMYND11** | Zinc Finger MYND-Type Containing 11 |
| **3D** | Three Dimensional |
| **7-AAD** | 7-Aminoactinomycin D |