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Original Research Paper

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HnRNPA1 ISOFORM BRING DIVERSITY IN GLIOMA CELL SURVIVAL

Vidhi Vashistha	
Sachin Bhardwaj	Dr. B. R Ambedkar Center for Biomedical Research, University of Delhi, Delhi 110007
Avinash Kumar	
Amit K.Yadav	Dr. B. R Ambedkar Center for Biomedical Research, University of Delhi, Delhi 110007.
Birendra K. Yadav	Rajiv Gandhi Cancer Institute & Research Centre, Rohni, Delhi, India.
Ajay K. Yadav*	Ass. Professor Dr. B. R Ambedkar Center for Biomedical Research, University of Delhi, Delhi 110007. *Corresponding Author

ABSTRACT Hypoxia response plays a critical impact on tumor growth and drug resistance development. Under hypoxic and non-hypoxic growth environment U87MG and A172 glioma cells behaved differently. The presence of hnRNPA1 isoforms variant showed critical regulatory step in directing glioma cell adaptability. Knocking hnRNPA1, distinctly affecting the hnRNPA1 isoforms in two glioma cell lines. U87 MG cells, in response two different siRNA against hnRNPA1, suppresses higher isoform of hnRNPA1 compare to lower isoforms, on contrary A172 responses predominantly with up regulation of hnRNPA1vII (higher isoform) only. Leading expression of HnRNPA1vII isoform down regulates phospho-ERK½ activation. Additionally, knock down of GSK3 alpha also enhances the generation of higher isoform of hnRNPA1 in non-hypoxic cells. Remarkably, cells grown under hypoxic condition, phospho-ERK½ activation was observed only in presence of lower abundant isoform of hnRNPA1. Furthermore, immunohistochemistry was conducted to recapitulate the moderate to weak expression of GSK3 alpha correlated with strong expression of hnRNPA1 in glioma hypoxic tumor. Nonetheless, selective knock down of hnRNPA1vII (higher form) and vI (lower isoform) in U87MG were examined, evidently the phospho-ERK½ expression was downregulated in hnRNPA1vI knock down, compared with higher phospho-ERK½ in hnRNPA1 higher isoform (vII) knock down was observed. Interestingly, knock down of hnRNPA1 vII (higher isoform) also reduces the expression of hnRNPA2/B1 and SF2/ASF1 (splice factor RNA binding protein), in U87 MG cells. In conclusion, selective targeting hnRNPA1 isoforms attenuate glioma cell metabolism, and promote therapeutic sensitization.

KEYWORDS : Hypoxia, hnRNPA1, pERK½, GSK3

INTRODUCTION

Glioma cell adaptation under various metabolic stresses or oxygen deprivation microenvironment initiates UPR (Unfolded protein response) signaling pathway[1], [2]. Hypoxia leads to endoplasmic reticulum stress deals with an aggregation of misfolded proteins[3], thus MAPK pathway plays a critical key role in cancer cell survival to overcome stress or induces cell death[4]. Constitutive or long term P-ERK½ activation potentiates the composition of transcription factor activator protein (AP-1) in turn, controls DNA binding and transcriptional activity[5], promotes cell survival. Strong activation P-ERK½ subject to growth factor stimulation leads to cell cycle arrest or differentiation[6]. ERK½ activation has a significant role over UPR signalling pathway as well as HIF-1 transcriptional activity[4], [7].

Sustained ERK½ activation promotes cyclin D1 expression[8] and also upregulate BiP[9]. ERK½ phosphorylate eIF4E[10], thus promoting cell proliferation as BiP chaperone clears the misfolded protein in ER lumen and eIF4E increases new protein synthesis under ER stress[9], [10]. HIF-1α subunit activity is also been regulated by ERK½, where ERK½ translocates to the nucleus and phosphorylate HIF-1α at its carboxy terminal domain to induces its transcriptional activity[7].

Additionally, under hypoxia state glioma cells overcome stress, majorly by acquiring metabolic shift from oxidative phosphorylation to glycolysis due to Isocitrate dehydrogenase mutation[11]; PKM1 to PKM2 (Pyruvate kinase) splice switching[12]; HK2 (Hexokinase) translocation to mitochondria[13], and acidification around tumor cells, thus builds tumor microenvironment. PKM1/PKM2 pyruvate kinase ratio is strongly correlated in response to an increase in expression of hnRNPA1, favors PKM2 synthesis[14].

Additionally, PI3K/AKT/mTOR pathway is constitutively active in hypoxia state and upregulates HIF-1α activity[15]. Phosphorylation of AKT at S473 and T308, further to downstream mediate GSK3, mTOR, FRAP, FOXO activation, promoting cell survival[16]–[18]. PI3K/AKT kinase inhibits Gsk3β through its phosphorylation at Ser9 residue[16].

From our previous publication, demonstrated GSK3β inhibition downregulate phospho-ERK½, induces apoptosis in glioma cells[19]. However, GSK3αsiRNA promotes cytoplasm to nuclear translocation of P-ERK1/2 and also controls hnRNPA1 expression[19], [20]. Furthermore, relevant hnRNPA1 isoform were observed in glioma cells and tissues from our studies, are confirmed by sanger sequencing (unpublished). HnRNPA1 isoform dependent activation of phospho-ERK ½ was examined, reveals us more effective in presence GSK3. Moreover, from our IHC studies moderate to weakly expressed glioma tissue mimics the hypoxia petridish cell culture model, showed a high expression of hnRNPA1. Therefore, considering hnRNPA1 specific isoform expression plays a key role, reprogramming survival signaling pathway.

MATERIAL & METHODS

Cell Culture Material:

U87 & A172 cells (Generous support from Prof. Subrata Sinha, N.B.R.C, Manesar, Gurgaon (India)) were propagated in Dulbecco's modified Eagle's medium (DMEM) (PAN Biotech) composition: 10% fetal bovine serum (PAN Biotech), 100 units/ml penicillin- streptomycin (PAN Biotech). Cells were grown under 37°C and humidified chamber under 5% Co_2.

Antibodies:

Western blotting of all essential proteins was probed using primary monoclonal antibodies anti-hnRNPA1 (Santa Cruz biotech. Inc.), anti-P-ERK½, anti-ERK½, anti-eIF4E (Santa

Cruz biotech. Inc) and anti-β-Tubulin (Abcam), followed with HRP conjugated mouse or Rabbit secondary antibodies (GeNei).

Stable Cell Lines:

We created U87 & A172 Gsk3β knock down stable cell lines in the lab compared with control knock down cells. These cell lines after attaining 60-70% confluency were transfected with Gsk3β Lentivirus (shRNA) (Santa Cruz Biotechnology, Inc.) as per manufacture's protocol. After 48-72hrs of transfection cells were selected with puromycin (Mammalian selection marker). For selection we added 5μg/ml of puromycin in the Gsk3β RNAi transfected cells. Transfected cells were grown in the puromycin containing selection medium. Confirmed knock down using lentiviral GSK3 alpha RNAi was reported from our previous study (30).

si RNA Transfection:

U87 & A72 CNTi and Gsk3βi (Stable cell line) cells were transfected next day after plating, with three different customized specific hnRNPA1 (10nM) siRNAs (Eurogentec), selectively against common 3'UTR and CDS region, and scrambled siRNA (Santa Cruz Biotechnology, Inc.) as per protocol. After 72hrs of transfection protein lysates were prepared in cell lysis buffer (Cell signalling technology) containing protease inhibitor (Abcam) and phosphatase inhibitor (Santa Cruz Biotechnology, Inc.). In all experiment siRNA mediated knock down efficiency was achieved ≥ 50% to 60 % using Interferrin transfection reagent from Polyplus Company. All experiment were performed at least three times, representative pictures were reported here.

Hypoxia Treatment:

In another experiment U87 & A172 cells were transfected next day after plating, with two different customized specific hnRNPA1 (10nM) siRNAs (Eurogentec) (3'UTR and CDS region) and scrambled siRNA (Santa Cruz Biotechnology, Inc.) as per protocol. After transfection cells were kept in hypoxic conditions (0.1% O_2) in the incubator. For hypoxic conditions, the chamber was charged with N_2 and CO_2. In the hypoxia incubator CO_2 concentration was 5% and rest N_2 was present to minimize the O_2 concentration to 0.1%. After 72hrs of transfection under hypoxia treatment protein lysates were prepared in cell lysis buffer (Cell signalling technology) containing protease inhibitor (Abcam) and phosphatase inhibitor (Santa Cruz Biotechnology, Inc.). In all experiment siRNA mediated knock down efficiency was achieved ≥ 50% to 60 % using Interferrin transfection reagent from Polyplus Company. All experiment were performed at least three times, representative pictures were reported here.

Immunoblotting:

Protein lysate were prepared in cell lysis buffer. Made protein lysate were quantified using Bradford reagent (Biorad). Westorn blotting was performed (30).

Immunohistochemistry:

Procedure related with studies on clinical samples were all approved by Institutional Human Ethical committee of Rajiv Gandhi Cancer Institute & Research Centre, Rohni, Delhi, India and Dr. B.R Ambedkar Center for Biomedical research, University of Delhi.

An immunohistochemical method using the anti-GSK3 alpha (EP793Y) antibody and Anti-hnRNP A1 antibody (9H10) separately in two different sides, was developed at Ventana Medical Systems. Sections (4 um) were cut from the FFPE blocks. Normal brain tissue used as a positive control. Automated IHC with the Ventana anti-GSK3 alpha (EP793Y)or hnRNPA1 antibody, was performed in a Benchmark Ultra staining module (Ventana Medical Systems, Tucson, AZ). In brief, the slides were deparaffinized using EZ prep followed by epitope retrieval (Cell conditioner no. 1, pH 8.5at 95 °C for sixty minutes). After retrieval the slides were blocked for peroxidase (OptiView peroxides inhibitor for four minutes, incubated for 48 min in 44 °C. UltraView Universal DAB Detection Kit was used for chromogenic detection and performed Ventana standard signal amplification, ultra Wash, counter- staining with one drop of Hematoxylin for 12 min and one drop of bluing reagent for 4 minutes. Staining intensities were recorded as weak, moderate, or strong. Faint diffuse staining, any type of isolated nuclear staining, weak staining of single interspersed cells, and staining of monocytes/macrophages were scored as negative. Moderate or strong staining was scored as positive.

Selective Knock Down Of hnRNPA1 vI And vII Followed With PI3 Kinase Inhibitor Treatment In U87 Glioma Cells:

U87 cells were transfected next day after seeding, with selective customized hnRNPA1 v1 and vII siRNA (10nM) (Eurogentec), selectively designed to target included intron sequence in between Exon 7 and Exon8 (higher hnRNPA1 isoform vII), and hnRNPA1 isoform vI were designed to target the junctional sequence in between Intron 7 and Exon 8: (siRNA sequence to target selectively hnRNPA1 vII :5'GCUAUGACAGCUAUAACAA dTdT 3'; and siRNA hnRNPA1vI: 5'UGGUAACGAUGGAAGCAAU dTdT 3'); were transfected in cells as per protocol. After 48 hrs of transfection cells were treated with PI3 Kinase inhibitor (Wortmannin) (10μM) for 24 hrs followed with protein lysate preparation. Protein lysate were prepared in Triton buffer (10nM Tris-cl, 100mM NaCl, 1mM EDTA, 1% TritonX-100) containing protease inhibitor (Abcam) and phosphatase inhibitor (Santa Cruz Biotechnology, Inc.). In all experiment siRNA mediated knock down efficiency was achieved around ≥ 50% to 60 % using Interferrin transfection reagent from Polyplus Company. All experiment were performed at least three times, representative pictures were reported here. After western blotting membranes were probed using different monoclonal antibodies like hnRNPA1, hnRNPA2B1, SF2/ASF1, BMI (Santa Cruz Biotechnology, Inc.), and phospho-ERK1/2, ERK½, phospho-AKT, α-Tubulin (Abcam) followed with HRP conjugated mouse or Rabbit secondary antibodies (GeNei).

RESULTS

HnRNPA1 and Gsk3α influences phospho- ERK ½ activation

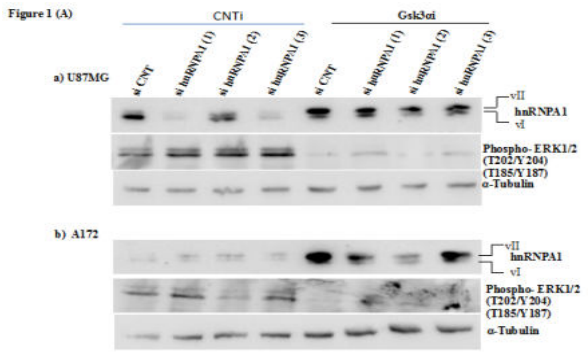
For this experiment three different siRNAs (sihnRNPA1(1), sihnRNPA1(2), and sihnRNPA1(3)) were used to knockdown hnRNPA1 in U87MG and A172 glioma cell lines. The amounts of phospho- ERK ½ and hnRNPA1 in different siRNA treated samples were compared through western blotting technique. Knockdown of hnRNPA1 was most efficient in the sihnRNPA1(1), and sihnRNPA1(3) treated samples. In U87MG cells, the hnRNPA1 knockdown samples showed an apparent upregulation of phosphor-ERK½ compared to the control (FIG-1A). This leads us to believe hnRNPA1 regulates phospho-ERK1/2 expression, however our previous finding showed GSK3β depletion, enhances both phospho-ERK1/2 and hnRNPA1 expression[20]. Furthermore, to study the effect of GSK3β on the observed impact of hnRNPA1 knockdown over phospho-ERK½, both Gsk3β and hnRNPA1 were silenced in another group of similar set of samples, Despite of co-knockdown of both Gsk3β and hnRNPA1, we showed that the hnRNPA1 was upregulated compared to the only hnRNPA1 knockdown cells, markedly a higher form of hnRNPA1 (vII), is more visible in GSK3β-i samples. Also, the additional silencing of Gsk3β obstructed the upregulation of phospho-ERK ½ caused by hnRNPA1 siRNA (Fig-1A). Hence, co-knockdown samples didn't show the evident increase in phospho-ERK½, as observed in samples with only hnRNPA1 knockdown cells. We sought hnRNPA1 higher form regulating phosphor-ERK½ downregulation in presence of Gsk3 alpha. So, we perform another set of experiment to ensure the role of Gsk3 on hnRNPA1 higher form upregulation through PI3K/AKT

kinase inhibition using LY294002, showed hnRNPA1 higher (transcription activation is more evident) form is more expressed upon Gsk3 activation (Supplementary Fig- 1,2,3). These results suggest a mediatory role of Gsk3 in the upregulation of phospho-ERK½ due to hnRNPA1 knockdown. Moreover, the similar line of experiments into the A172 cell lines conferred similar result reproduces with higher form of hnRNPA1 in GSK3 knockdown. Though, we didn't find much expression of hnRNPA1 in the siRNA control samples of the A172 cell line. The hnRNPA1 and GSK3 co-knockdown samples showed abundant expression of higher form of hnRNPA1 and didn't exhibit the upregulation of phosphor-ERK½, as observed in the only hnRNPA1 knockdown A172 cells.

HnRNPA1 Knockdown Under Hypoxic Growth Conditions

In this study, siRNAs sihnRNPA1(1) and sihnRNPA1(3) were chosen, considering their knockdown efficiency. Our examination of phospho-ERK½ expression in U87MG cells revealed a similar but more prominent pattern of upregulation of phospho-ERK½ in hnRNPA1 knockdown samples compared to siRNA control. These results stand consistent with our previous studies of U87MG and A172 hnRNPA1 knockdown cells in non- hypoxic conditions, except the prominent expression of phosphor-ERK½ was observed in hnRNPA1 knockdown cells. Moreover, hnRNPA1 knockdown U87MG cells showed negative expression of eIF4E compared to si RNA Control U87 MG. On other hand, A172 cells in control siRNA had upregulated expression of phospho-ERK½ in hypoxia; this finding is consistent with previous studies that have demonstrated the role of phospho-ERK 1/2 in hypoxia[21]. Also, a significant decrease in expression of phosphor-ERK1/2 was observed in the hnRNPA1 knockdown cells compared to the control. This significant decrease in expression of phospho-ERK1/2 has been seen with increase in eIF4E expression. However, higher isoform of hnRNPA1 were more visible in A172 glioma cell line (Fig- 1B).

Though siRNA used in our experiments were not specific for any selective isoform of hnRNPA1. Therefore, hnRNPA1 siRNA should have knocked down both the mRNA transcripts of the variant isoforms equally. Yet our results indicate that in the U87 cell lines in the hypoxic environment, hnRNPA1 siRNAs were able to knock down the hnRNPA1 vII completely but were less down regulation of hnRNPA1 vI isoform in comparison of siCNT. Opposing to these results, in A172 cells, the hnRNPA1 vI isoform (lower form) was comparatively silenced, whereas hnRNPA1 vII (higher form) was comparatively more expressed (FIG-1B). These opposing results in different cell lines are not unprecedented but are expected, given that high heterogeneity in glioma cells, with high hnRNPA1 polymorphism as expected stored in 40S ribosome[22], could be one reason. The regulatory switch control driven by hnRNPA1 isoform against phosphoERK1/2 in hypoxia was not explored before. Relevant information with decrease in expression of phospho-ERK ½ in hnRNPA1 knockdown samples can be attributed to the specific hnRNPA1 isoforms were discussed subsequently in next section.



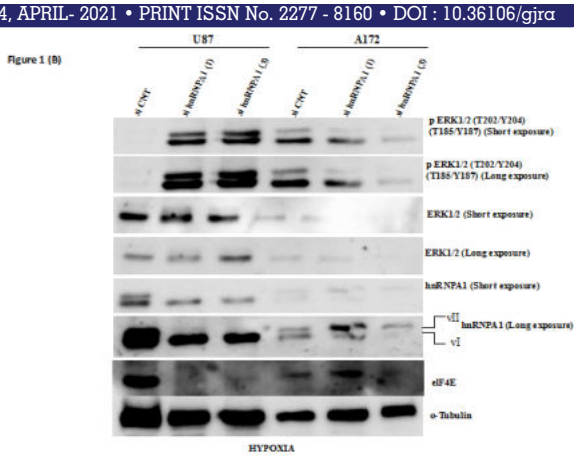


Fig 1:- hnRNPA1regulate phospho-ERK½ Differently Under Normoxia And Hypoxia:

A.CNT-i and Gsk3α lentiviral knock down glioma cell line were co-tra nsfected with three different hnRNPA1 siRNA were compared a) U87, b) A172

B. Under hypoxia microenvironment, expression of phosphor-ERK½, eIF4E level were analyzed in two different hnRNPA1 siRNA, transfected U87MG and A172 glioma cells

Immunohistochemistry

Expression of GSK3 alpha was co-related with the expression of hnRNPA1. Positive experiment control for Brain tissue section compared with (n=13) glioma paraffin embedded tissue slides. Expression of GSK3 alpha were scored as High, moderate, weak and negative in glioma tissue slide sections. Out of (n=13) tumor tissue slides (n=3, moderate; n=3 negative; n=7, weak) expresses GSK3alpha and expression of hnRNPA1 found high in all the thirteen GBM tissues. Correlation analysis was obtained - 0.78, p< .05, after counting the number of positive spots at five different sites in each tissue slide stained with GSK3 alpha or hnRNPA1 separately(FIG-2).

Figure-2

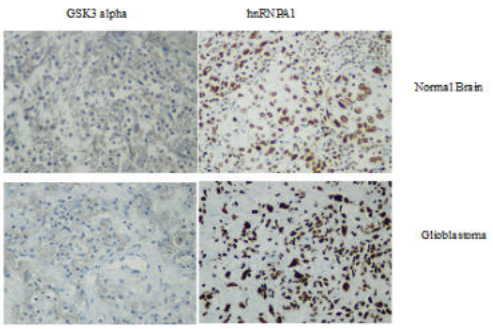


Fig 2:- Immunohistochemistry (IHC) analysis of glioma tissue section show less expression of Gsk3α with more hnRNPA1 protein., Picture were of scale bar 20m.

Selective Knock Down Of hnRNPA1 vI And vII In U87 MG Cell Line:

Knock down hnRNPA1 vII lends glioma cells to decrease the expression of hnRNPA2B1, SF2/ASF1 and Bmi-1, a Polycomb family protein act as transcription repressor; phospho-ERK½ activity was comparatively increase in hnRNPA1 vII knock down cells. Knock down of hnRNPA1 vII, however showed increase in post-translational modified hnRNPA1vI isoform, is yet to pursue further (Fig- 3A). The post-translational modified form of hnRNPA1 vI (lower isoform) visible in hnRNPA1 vII knock down, was disappeared in wortmannin (10M) treated as showed in Fig- 3B. This suggest hnRNPA1 vI (lower isoform) is more active and prevalent in absence of hnRNPA1 vI1.

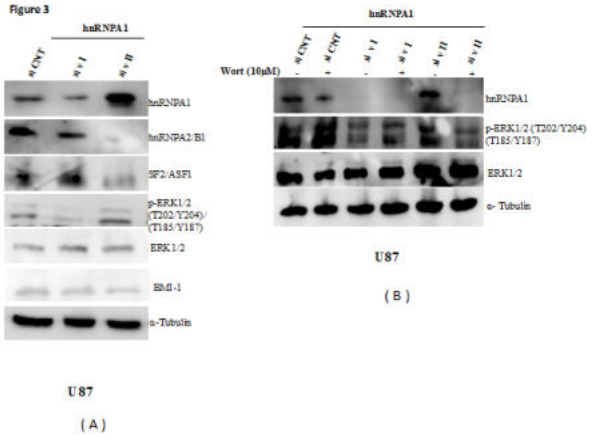


Fig 3:- Selective Knockdown Of hnRNPA1 vI And vII In U87 MG Glioma Cell Lines:

A. Knockdown of hnRNPA1 using specific siRNA against vI and vII in U87 glioma cell line were performed. Expression of hnRNP2/B1, SF2/ASF1, pERK1/2, Bmi-1 were analyzed using western blotting.

B. Knockdown of hnRNPA1 using specific siRNA for vI and vII in U87 glioma cell lines in absence and presence of Wortmannin (10μM) were performed.

DISCUSSION

Under hypoxia state along with nutrient deprivation, angiogenesis processing regulated by overexpressing genes like vascular endothelial growth factor (VEGF)[23], angiopoeitin-2[24], fibroblast growth factor (FGF) ., enhances angiogenesis or anti- angiogenesis, deciding the fate of the cell, such as VEGF-A, a well-known angiogenic gene adapts hypoxia in presence of SRSF1 overexpression, producing VEGF-A_{xxx}a alternative spliced variant enhances angiogenesis[25], also hnRNP L(splice factor) results increase in VEGF-A expression[26], [27]. Hypoxia, also predominantly leads to increase in hnRNPA1 expression to facilitate gene reprogramming and its transcript processing; such as PKM splicing process directly by hnRNPA1[28], splicing of MAX (Myc associated factor X) by hnRNPA1 in presence of constitutive active EGFR vIII prefers glycolytic adaptation in hypoxic gliomas. HnRNPA1 (splice factor) is a member of the hnRNPA/B family protein promotes stress granule formation, to delay the transcription process under varied stress condition, act as store house[30], [31]. hnRNPA1 gene encodes, abundantly expressed of hnRNPA1-a of size 320 amino acid and a spliced variant hnRNPA1-b of size 372 amino acids[32]. These two splice variants varyingly as per affinity binds to pre-mRNA for transcript processing, may have putative long term or short-term transcription processing. Overexpressed SF2/ASF1(splicing enhancer) in presence of hnRNPA1 (splicing suppressor), competes for its binding at the 5' splice site[33], opposes pre-mRNA splicing by displacing hnRNPA1 promotes tumorogenesis[34]. Therefore, abrupt imbalance in between hnRNPA1 and SF2/ASF1 leads to cell transformation.

Our study proposes that knockdown of GSK3 alpha effectively upregulates the expression of hnRNPA1 vII (Higher isoform) causes pERK½ inactivation in U87MG and A172 cell lines, on contrary A172 glioma cell lines grown in hypoxic condition, has comparatively less total hnRNPA1 expression activates pERK½ . However, U87MG with endogenous high expression of hnRNPA1, only activates pERK½, with subsequent loss of hnRNPA1 vII (higher isoform). This shows higher the comparative expression of hnRNPA1 higher form vII/vI lower isoform ratio causes loss of pERK½ activity. Moreover, in U87 and A172 glioma cells after using two different siRNA against hnRNPA1 irrespective of hnRNPA1 isoform, apparently developed different hnRNPA1 isoform, only because of due to stored hnRNPA1 polymorphism in 40S ribosome, or in stress granules. We are working on two spliced isoform of hnRNPA1 vII and vI, both the spliced variant isoform of hnRNPA1 were confirmed by sanger sequencing.

Moreover, to validate the hypothesis, selectively transfecting siRNA against hnRNPA1 vII transcript promotes moderate pERK½ activation, in absence of GSK3 alpha knock down. Knock down of hnRNPA1 vII also leads to loss of SF2/ASF1, hnRNPA2/B1, and Bmi-1. Due to inherent heterogeneity among in between glioma cells, as showed difference in expression of GSK3 alpha varies in glioma cell lines[20]. Our experiment showed loss of GSK3 alpha inhibits pERK½ activation, in the presence of hnRNPA1 vII, mimics moderate to weak expression of GSK3 alpha in glioma tissues, showed strong expression of hnRNPA1, probably is hnRNPA1 vII dependent, is not yet confirm in vivo. In cancer, Ras/Raf Kinase was proposed to activate phospho-ERK½, gene amplification of A-Raf kinase was showed in Astrocytoma[35]. Alternative spliced variant form of A-Raf kinase, A-Raf (short form) devoid of kinase domain but have Ras binding domain, inhibits RAS induced cell transformation[36]. c-Myc transcriptionally promotes hnRNP's family gene [37], out of which hnRNP A2 and hnRNP H overexpression in hepatic carcinoma showed up regulation in phospho-ERK½ activity hence promote tumorigenesis[36], [38]. On the other hand, phospho-ERK½ was downregulated in hnRNPA2 and hnRNPH knock down cells subsequently, promote splice switching from A-Raf kinase full-length gene to A-Raf (Short form) devoid of kinase domain, required for subsequent phospho-ERK½ activation[36], [38]. In fact, hnRNPA2 spliced isoforms differently regulate Ras/Raf/MEK mediated phospho-ERK½ activation[38].Additionally, suffices the predictive function of GSK3 in hypoxia, leading to HIF-1alpha (hypoxia inducible factor) destabilization [39].

Relevant schematic presentation (Fig;4)

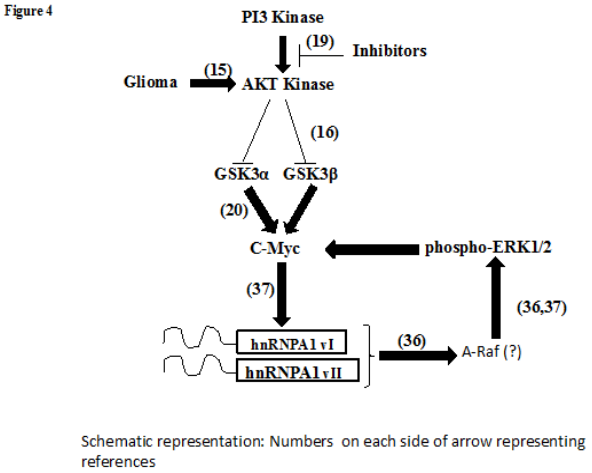


Fig: 4 Flow chart showing PI3k/Akt mediated GSK3 inhibition resulting in phospho-ERK activation through hnRNPA1 isoforms under c-Myc regulation.

Declaration: No conflict of Interest

Human Ethical Approval:
Approved by Institutional Human Ethical committee

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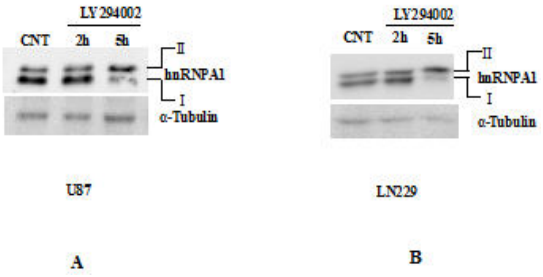
Authors Contribution:
V.V, S.B, Amit K.Y conducted experiment; B.Y did experiment on Glioma tumour tissues and its analysis; A.K.Y conceived, designed experiments and drafted manuscript.

Availability Of Data Material:
Data will be available on request

Consent To Participate:
Glioma tumor tissue silde for analysis approved by Institutional human Ethical Committee (I.H.E.C).

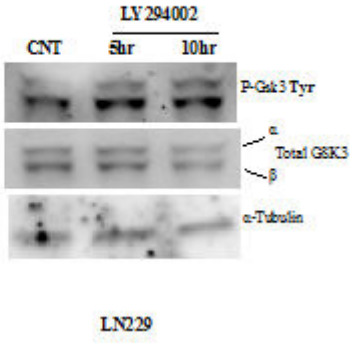
Consent For Publication: Not applicable

Supplementary Figure – 1



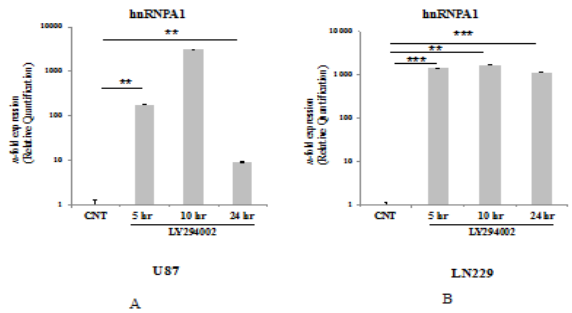
Supplementary Fig 1:- PI3K/AKT inhibition using LY294002 inhibitor allows expression of hnRNPA1 higher form (vII) showed in western blotting

Supplementary Figure - 2



Supplementary Fig 2:- LN229 cells were treated with PI3K/AKT inhibitor LY294002 (10uM) for 5hr and 10hr

Supplementary Figure - 3



Supplementary Fig 3:- Data analyzed using quantitative real time PCR, after cells treated with PI3K/AKT inhibitor LY294002 showing n-fold increase in hnRNPA1 expression:

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