Clinical and therapeutic significance of epithelial to mesenchymal transition (EMT) like process in glioma

Thesis submitted to the University of Hyderabad
for the degree of

Doctor of Philosophy

in

Biotechnology and Bioinformatics

By

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September, 2018

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DECLARATION

The research work presented in the thesis entitled **"Clinical and therapeutic significance of epithelial to mesenchymal transition like process in glioma"** has been carried out by me in the Department of Biotechnology and Bioinformatics, School of Life Sciences, University of Hyderabad, Hyderabad, under the guidance of Prof. P. Prakash Babu. I hereby declare that this work is original and has not been submitted in part or full for any other degree or diploma of any other University or Institution.

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CERTIFICATE

This is to certify that the thesis entitled **"Clinical and therapeutic significance of epithelial to mesenchymal transition like process in glioma"** submitted by Mr. Deepak Babu bearing registration number 10LTPM05 in partial fulfillment of the requirements for award of Doctor of Philosophy in the Department of Biotechnology and Bioinformatics, School of Life Sciences is a bonafide work carried out by him under my supervision and guidance.

This thesis is free from plagiarism and has not been submitted previously in part or in full to this or any other University or Institution for award of any degree or diploma.

Parts of this thesis have been:

A. Published and/or under Consideration in the following journals:

1. Ravindra Pramod Deshpande, Deepak Babu, Manas Panigrahi, Chandra Sekhar Y.B.V.K, Phanithi Prakash Babu. Brain tumors incidences and a retrospective clinical analysis from a tertiary hospital in India. J Neurooncol. 2016;129(2):383-7. ISSN: 1559-1182.
2. Deepak Babu, Anwita Mudiraj, Chinatal Ramulu, Manas Panigrahi, Phanithi Prakash Babu. Portrayal of EMT like process in human glioma and its impediment by rabeprazole to combat glioma growth and temozolomide resistance. Under communication in Cellular and Molecular life Science (Manuscript ID: CMLS-D-18-01038).
3. Deepak Babu, Chinatal Ramulu, Manas Panigrahi, Phanithi Prakash

Babu.Breast cancer metastasis suppressor 1 aggravates glioblastoma (GBM) malignancy in *mut*P53 GBM. Under communication in The Journal of Pathology (Manuscript ID: ID 18-533).

4. Deepak Babu, Chinatal Ramulu, Manas Panigrahi, Phanithi Prakash Babu. Therapeutic implication of plumbagin in CTTN-EGFR modulated astrocytoma malignancy. Under communication in Acta-Pharmaceutica Sinica-B (Manuscript ID: APSB_2018_599).
5. Deepak Babu[#], Khamusha valli[#], Phanithi Prakash Babu. Pantoprazole induces mitochondrial apoptosis and attenuates NF-κB signaling in glioma. Received minor comments; Cellular and Molecular Neuroscience (Manuscript ID: CEMN-D-18-00163).

Manuscripts under preparation:

6. Deepak Babu, Anwita Mudiraj, Manas Panigrahi, Phanithi Prakash Babu. Rabeprazole curb glioma growth and malignancy- a preclinical evidence.
7. Deepak Babu, Manas Panigrahi, Phanithi Prakash Babu. BRMS1 attenuate glioblastoma invasion and TMZ resistance by repressing EMT pathway in *mut*P53 glioblastoma.

B. Presented in the following conferences:

1. Presented poster in Indo-Taiwan Joint Interdisciplinary Neuroscience Symposium-2014 held during Jan 9-10, 2014 at School of Life Sciences, University of Hyderabad, India. (International)
2. Presented oral in 29th annual meeting of SNCI-ACNN 2015 from 19th to 21st Dec 2015 organized by Department of Biomedical Engineering and Computer Centre, North-Eastern Hill University, Shillong, India. (National)
3. Presented poster in BIO-QUEST 2016 Symposium held at School of Life Sciences, University of Hyderabad on 29-30th Sept 2015. (National)

4. Presented poster in 31th annual meeting of SNCI 2017 from 20th to 22nd Sep 2017 organized by Institute of Science, Banaras Hindu University, Varanasi, India. (National)
5. Presented poster in BIO-QUEST 2017 Symposium held at School of Life Sciences, University of Hyderabad on 12-13th Oct. 2017 (Best Poster Award). (National)
6. Presented poster during International conference on 'Innovations in Pharma and Biopharma Industry: Challenges and Opportunities for Academy and Industry (ICIPBI)-2017' held at School of Life Sciences, University of Hyderabad on 20th-22nd Dec. 2017 (Junior Scientist Award-Best Poster). (International)

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This work is dedicated to my beloved parents whose patience and sacrifices will remain my inspiration throughout my life

Acknowledgements

First and foremost, I would like to acknowledge my supervisor Prof. P. Prakash Babu for allowing me to work on an exciting aspect of cancer biology. His constant support, stimulating guidance and encouragement enabled me to complete research work successfully. I am really thankful to him for being open to new ideas and the freedom he provided me to carry out my research work.

I am grateful to the doctoral committee members Dr. Nooruddin Khan and Dr. Ravi Kumar Gutti for their constant support and valuable suggestions during the entire thesis work.

Dr. Manas Panigrahi, Dr. Chandrasekhar Y.B.V.K (Neurosurgery Department,) Dr. Satish Rao and Dr Sailaja (Pathology Department) are highly acknowledged for providing clinical samples from Krishna Institute of Medical Sciences for the research work.

I would like to thank the present and former heads of the Department of Biotechnology and Bioinformatics and present and former deans of School of Life sciences for providing the departmental/School facilities.

I am thankful to all the non-teaching staff of the department and school specially Mr. Rajshekhar, Mr. Shekhar, Mr.Murthy and our lab assistant Mr. Muthaym and Mr. Chandan for their help in doing all the official works.

Dr. Anwita Mudiraj and Dr. Chintal Ramulu need to be specially acknowledged for the constant support and guidance during my thesis work.

All the present and former members of Neuroscience laboratory are highly appreciated for creating wonderful environment in lab and outside which has helped in being stress free all throughout my stay in Hyderabad. The senior lab members Dr. Prabhaker Dr. Ramulu, Dr. Khamushavali, Dr. Suraj, Dr. Nobel, Dr. Apoorv, Mr. Kartik, have been so great from the very first day that I never felt, new to the group and have trained me in different ways which helped me to carry out my research in the lab. I would like to thank Postdoctoral fellows of the lab, Dr. Raghu G, Dr. Vimal, Dr. Venkata, Dr. Parimala for their help and guidance's during this work. I

express my heartfelt gratitude to Dr. Ravindra-Manathker, Mr. Sireesh, Mr. Praveen, Mr. Naidu, Mr. Kishore, Ms. Ruchi, Ms. Neera, Ms. Subhangi, and Mr. Rahul for helping me one way or the other during this work. I would also like to thank the M.Sc project students Mr. Tahir and Mr. Sulemaan who worked with me during the period.

Dr. Nishant, Dr. Arif, Dr. Savita, Dr. Shivendra, Dr. Kishore, Dr. Narendra, Dr. Balakrishna need a special mention for their help in my work as well as being part of all our fun time I express heartfelt thanks to them.

I must thank my graduation friends Mr. Niraj, Mr. Arvind, Mr. Pankaj, Mr. Sunil, Mr. Mohit and Mr. Divya Prakash for constant help and support. I express gratitude to them for always being there for me.

I am indebted to my friend and roommate Amit Ranjan for all the fond memories and always standing by my side and sharing a great relationship as compassionate friend. I will always cherish the warmth shown by him.

I am also grateful to my classmate friends in the university and department Dr. Nidhi, Dr. Prashant, Dr. Nikhil, Ms. Bramhini and Dr. Tony Thomas for their help and support in my work and sharing sweet memories. I express heartfelt thanks to them.

I am extremely blessed to have a lovely family and would like to thank all, especially my grand-parents and parents for infallible love, care and faith in me which has boosted me always and helped me to carry out my work with positive note.

A special gratitude to my sisters Ms. Sushila, Ms. Sadhana, Ms. Aradhana, Ms. Anamika and brother Mr. P.S. Bandhu and Mr. Shyam Narayn for their love and affection.

I would like to thank each and every individual for the slightest of help they would have done during the entire work.

I would like to thank Department of Science & Technology (DST-India), Department of Biotechnology (DBT-India), Indian Council of Medical Sciences (ICMR-India) and University with potential for excellence (UPE-India) for funding support to the lab. Department of Biotechnology (DBT) is highly acknowledged for the financial support as Junior and Senior research fellowship.

Last but the most important one, All that I cherish today is the grace of my Guru and God. I thank the Almighty for granting me the strength, wisdom, knowledge and showering his blessings.

Deepak

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Abbreviation

ASR	Age standardized incidence or mortality Rate
BBB	Blood brain barrier
Bcl-2	B-cell lymphoma protein-2
Bax	Bcl-2 associated X protein
BRMS1	Breast cancer metastasis suppressor gene 1
CDKN2A	Cyclin-dependent kinase inhibitor 2A
CNS	Central nervous system
CT	Computed tomography
DCFDA	2',7' –Dichlorofluorescin diacetate
EDTA	Ethylene diamine tetra acetate
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EMT	Epithelial to mesenchymal transition
FACS	Fluorescence activated cell sorting
GBM	Glioblastoma multiforme
GFAP	Glial fibrillary acidic protein
GMT	Glial to mesenchymal transition
GSK3β	Glycogen synthase kinase 3β
IDH1	Isocitrate dehydrogenase 1 (NADP+), soluble
IF	Immunofluorescence
IHC	Immunohistochemistry
ING4	Inhibitor of growth protein 4
HIF	Hypoxia-inducible factor
MAPK	Mitogen activated protein kinase

MDM2	Mouse double minute-2 protein
MRI	Magnetic resonance imaging
MSG	Metastasis suppressor gene
***mut* P53**	Mutant P53
mTOR	Mammalian target of rapamycin
NF-κB	Nuclear factor κB
PARP	Poly (ADP-ribosyl) polymerase
PI	Propidium iodide
PFA	Para-formaldehyde
PI3K	Phospho-inositol-3-kinase
PPI	Proton pump inhibitor
PTEN	Phosphatase tensin homologue
RH-123	Rhodamine 123
RIPA	Radioimmunoprecipitation assay
ROS	Reactive oxygen species
RT- qPCR	Reversed transcribed-quantitative PCR
STAT3	Signal transducer and activator of transcription 3
TFs	Transcription factors
TICs	Tumor initiating Cells
TMZ	Temozolomide
TNFα	Tumor necrosis factor α
TUNEL	Terminal deoxynucleotidyl transferase (TdT) dUTP nick-end labeling
UPA	Urokinase plasminogen activator
WHO	World health organization
***wt*P53**	wild P53
Zo-1	Zonula occludens 1

Chapter 1

Introduction

General Introduction

The cell proliferation and cell differentiation is meticulously regulated process in our body (Ruijtenberg and van den Heuvel 2016). Alteration or any breakdown in these regulatory mechanisms imposes uncontrolled and abnormal cell growth ensuing formation of cell mass or lump widely known as tumors (de Oliveira et al. 2010; Hanahan and Weinberg 2000). Tumors can be categorized into two forms: benign and malignant. Benign tumors are non-migratory and non-invading form confined to their original location **(Figure 1)** (de Oliveira et al. 2010; Louis et al. 2007). The malignant tumors are rapidly dividing cell lump with migratory and invasive property. It can spread to other site of the same organ or other body parts, and is able to form a new tumor there; it is the real threat of cancer **(Figure 1)**.

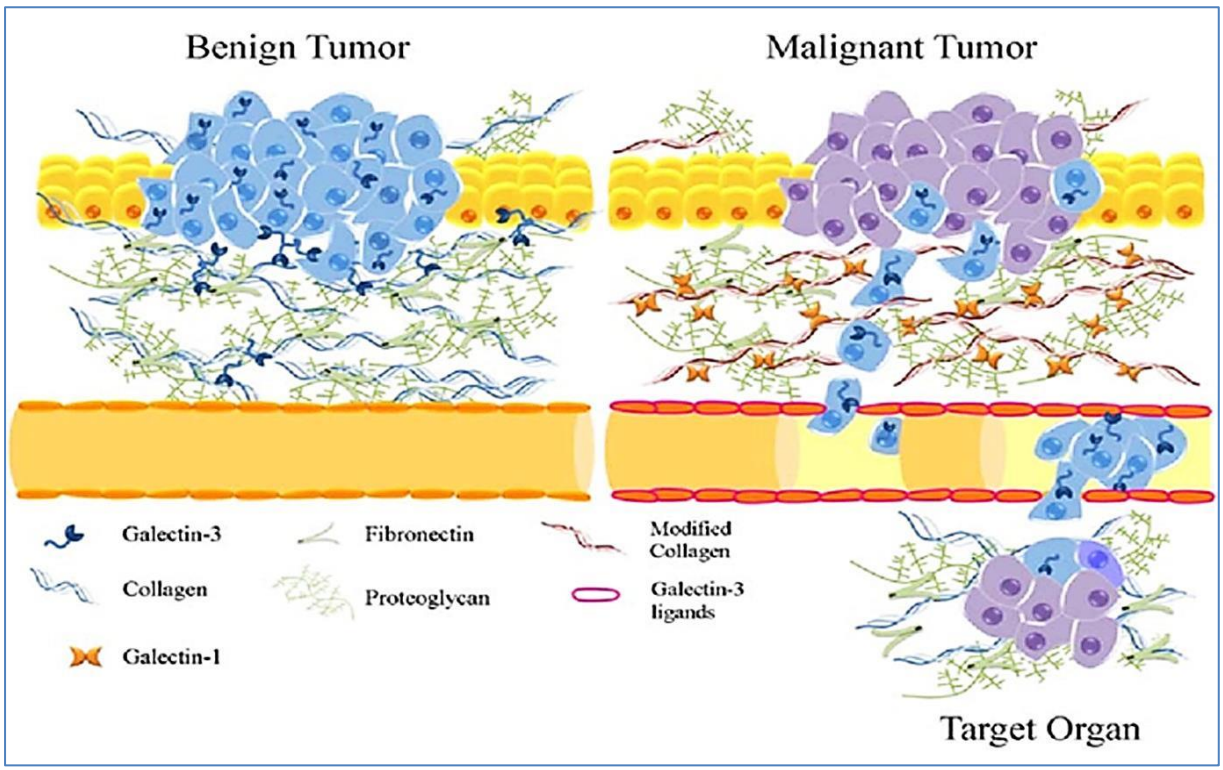

Figure 1. Schematic representation of benign and malignant tumor [de Oliveira et al. 2010]

Brain tumors

The brain is a vital part of central nervous system (CNS) responsible for information processing through nerve impulses and thereby controlling and coordinating the body functions (Jones 2012). Briefly, brain constitutes neurons, glial cell and meninges, and alteration in cellular machinery of these cells leads to neuronal cells derived neoplasm 'neuroblastoma', glial cell derived neoplasm 'glioma' and meninges derived neoplasm 'meningioma' (Gladson et al. 2010; Louis et al. 2007). Neuroblastoma and meningioma are beyond the scope of the present study.

Glioma

Gliomas are the frequently reported CNS tumors derived from glial or precursor cells **(Figure 2A)**. It contributes 30% of all primary brain tumors, 80% of which are malignant glioma (Louis et al. 2007; Ostrom et al. 2017). On the basis of cell of origin, gliomas are classified into astrocytoma, oligodendrogliaoma, ependymoma and mixed glioma. Astrocytomas including glioblastoma (GBM) account 75% among all form of glioma **(Figure 2B)** (Huse and Holland 2010; Louis et al. 2007; Ostrom et al. 2017).

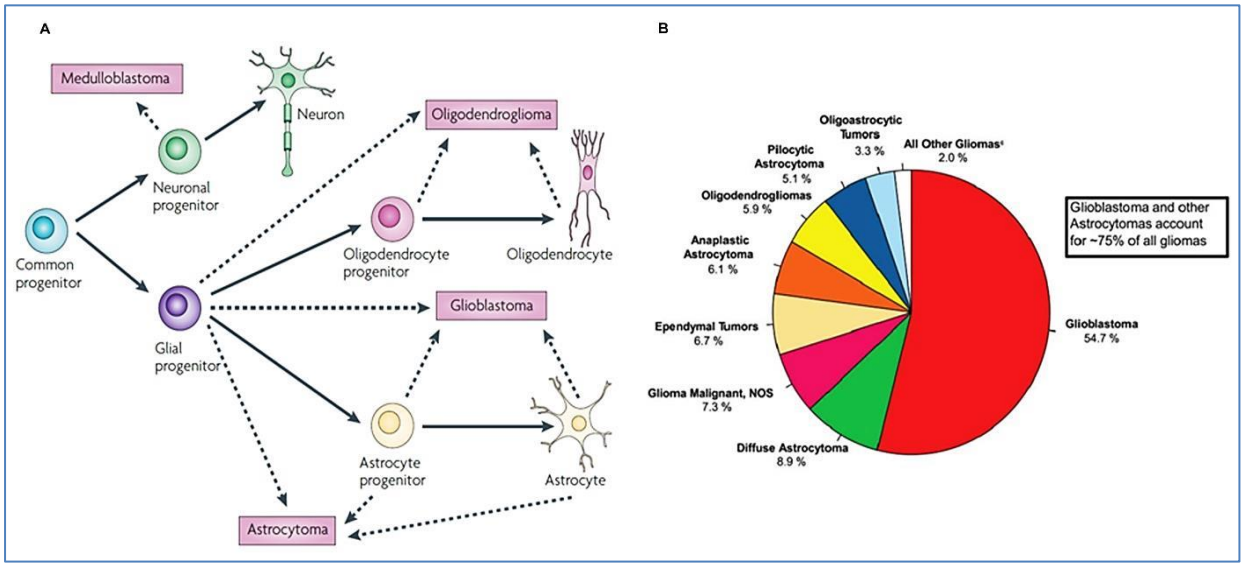

Figure 2. (A) Neuroglial lineage tree [Huse and Holland 2010] (B) Statistical distribution of glioma [Ostrom et al. 2017]

World Health Organization (WHO) Classification of Gliomas

In 2007, WHO histopathologically classified astrocytoma into four grades such as grade I (pilocytic astrocytoma), grade II (diffuse astrocytoma), grade III (anaplastic astrocytoma) and grade IV (glioblastoma multiforme)(Louis et al. 2007).

1. Grade I (GI) or pilocytic astrocytomas are biologically benign tumors **(Figure 3)**, proper diagnostic and total tumor resection of these results into complete cure.

2. Grade II (GII) or diffuse astrocytomas are biologically low-grade malignant tumors with diffuse infiltrative features **(Figure 3)** challenging/complicating complete tumor resection and thus require longer clinical observations (Furnari et al. 2007; Louis et al. 2007).

3. Grade III (GIII) or anaplastic astrocytomas are malignant tumor characterized by cellular or nuclear atypia with high cell proliferation and differentiations over GII tumors **(Figure 3)**. Because of its fast growing behaviour these tumors quickly progress into grade IV.

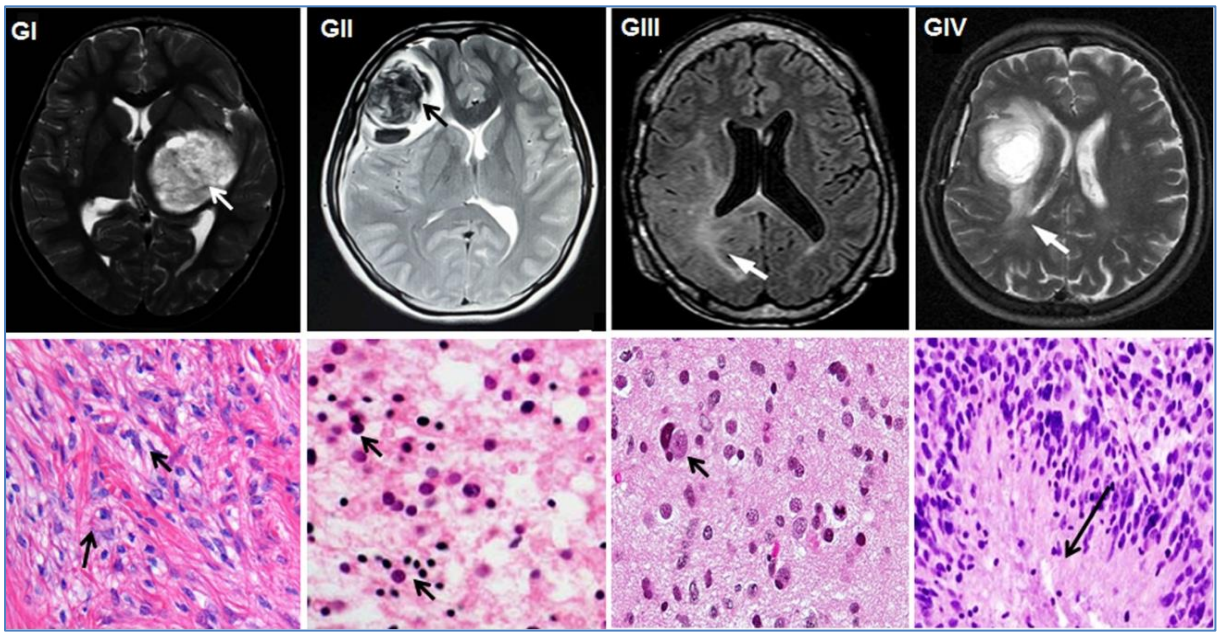

Figure 3. (A) MRI and (B) Histopathological imaging of astrocytoma grades representing typical histological features [Murakami R et al 2008; Yuan Rong et al 2006]

4. Grade IV (GIV) or glioblastoma (GBM) are highly malignant tumors characterized by increased cellular or vascular proliferation with necrotic cell death in tumor core region **(Figure 3)** (Furnari et al. 2007; Louis et al. 2007).

GIV tumors can further be divided into two forms primary glioblastoma and secondary glioblastoma on the basis of clinical and molecular presentations (Furnari et al. 2007). Primary glioblastoma are de novo form diagnosed without any history of low or high grade of astrocytoma (Furnari et al. 2007; Weller et al. 2015). It is mostly reported at older age (≥60 years) and account more than 90% of all GBM cases. Secondary glioblastoma are successive form of GBM. It is derived from a pre-existing low grade astrocytoma and mostly affects middle age adults (35-50years) (Furnari et al. 2007). In primary GBM, most frequently observed genetic alterations are amplification or mutation of EGFR and MDM2 gene and loss or inactivation of PTEN and LOH 10p and 10q **(Figure 4)**(Furnari et al. 2007; Louis et al. 2007; Weller et al. 2015). Hypermethylation in promoter region of MGMT gene are found in 36% of primary GBM and 75% of secondary GBM. PTEN mutation and loss of 10q or P53 is distinctive feature of secondary GBM **(Figure 4)** (Furnari et al. 2007).

In spite of the similar histology, molecular analyses have shown that both GBM subtypes are remarkably different at genetic levels, which are one of the major factors for ambiguous clinical outcome due to implication of same treatment regimen for both forms (Furnari et al. 2007; Rampling et al. 2004). This reflects the significance of molecular impact and urge for the personnel therapy. Moreover, in 2016, WHO introduced a new classification system for CNS tumors. In this classification system, the status of three signature gene isocitrate dehydrogenase (*IDH*), chromosomal 1p/19q codeletion and ATP-dependent helicase-*ATRX* are majorly utilized in addition to the tumor histology to categorized CNS tumors (Louis et al. 2016).

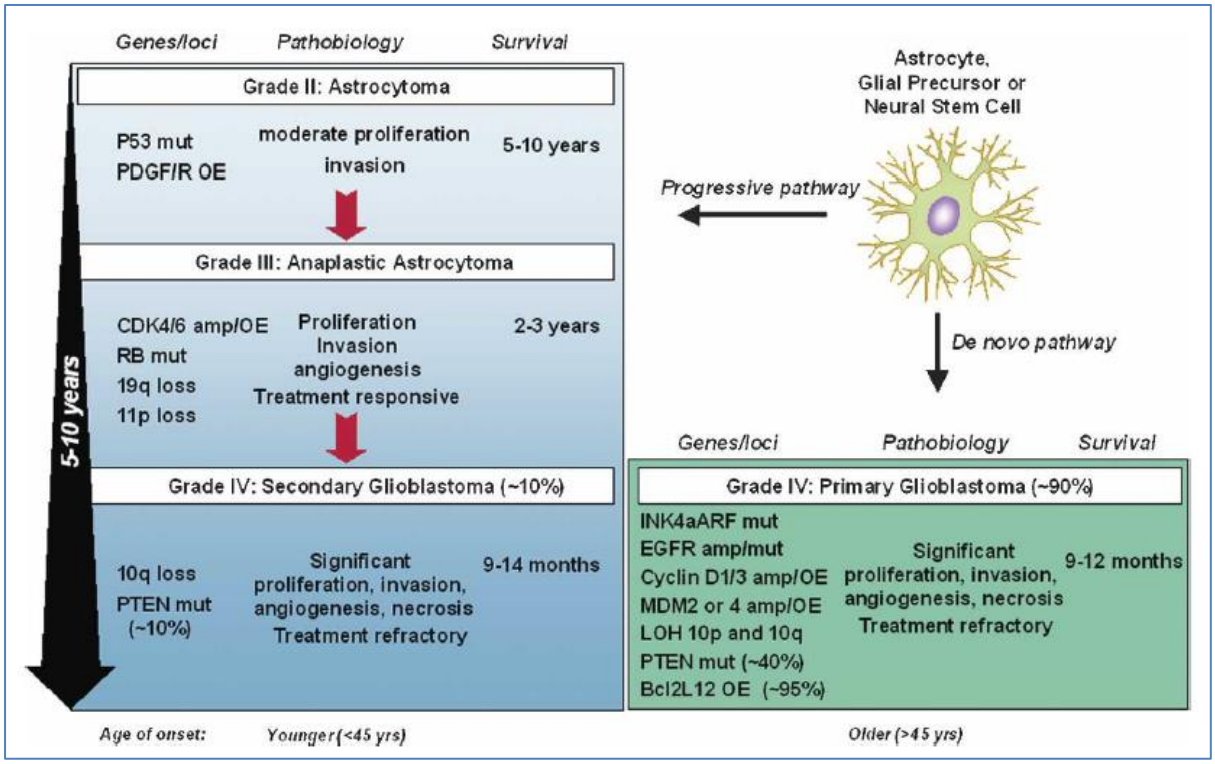

Figure 4. Chromosomal and genetic alteration in genesis of glioblastoma [Furnari et al. 2007]

Global incidences of brain tumors

The worldwide incidence rate of brain tumors in 2012, using the world standard population, was 3.4 per 100,000 with a total estimate of 256,213 individuals in 2012 (Leece et al. 2017). The incidence rates were higher in developed countries (5.1 per 100,000) than in less developed countries/developing countries (3.0 per 100,000); Central brain tumor registry United States (CBTRUS) Fact Sheet-2016 (Ostrom et al. 2017). The total incidence rates of all malignant tumors are significantly diverse in different geographical location for example highest incidences were reported in Southern Europe and lowest in Southeast Asia **(Figure 5)**(Leece et al. 2017).

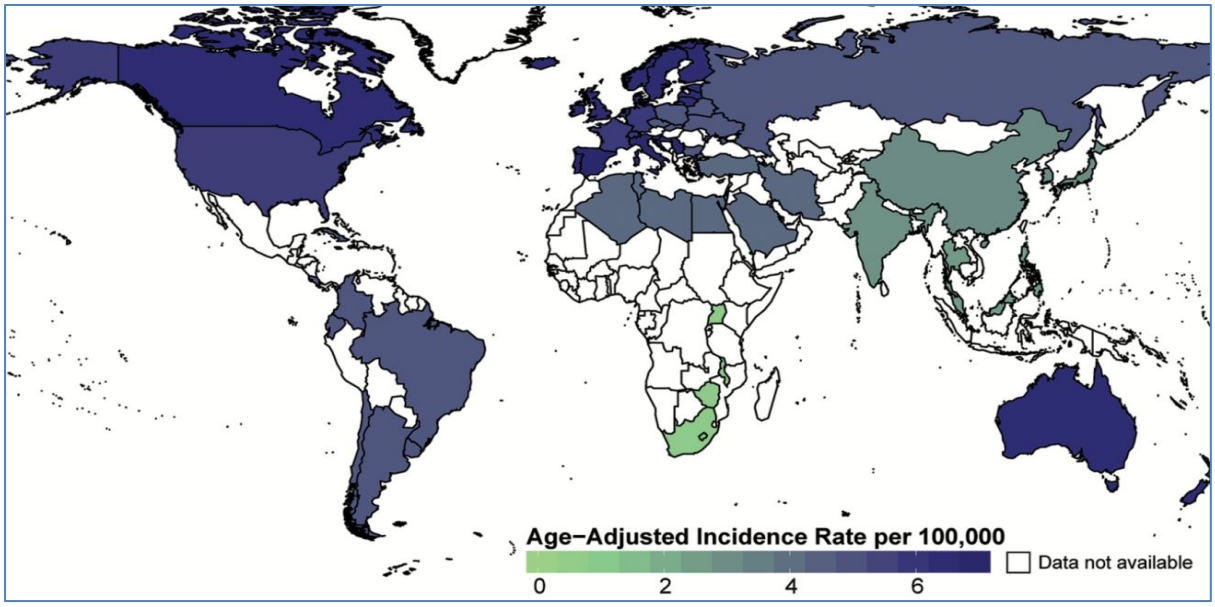

Figure 5. Global incidence of malignant brain tumors [Leece et al. 2017]

The total brain tumor incidences in India is lacking because there is no such agency for the monitoring of cancer incidences in India, However recently Indian Council of Medical Research (ICMR)- India has formed a national cancer registry (http://www.ncrpindia.org) to monitor cancer incidences across India. In 2016, we reported 1232 brain tumor incidences from a tertiary hospital in India during the period of 2009-2014 (Deshpande et al. 2016). In total brain tumor incidences astrocytoma accounts 53% among all tumors and 48% are malignant astrocytomas **(Figure 6)** (Deshpande et al. 2016) which signifies its prevalence in India.

The Current standard of care for glioma

The current standard of care for the low grade gliomas are surgical resection followed by regular therapeutic interventions. The therapeutics regimen for high grade glioma particularly GBMs are varied and completely based on clinical and molecular presentation (Hanif et al. 2017; Rampling et al. 2004). The current standard of care for GBMs is the summation of regular therapeutic treatment and supportive care of GBM patients (Hanif et al. 2017; Rampling et al. 2004).

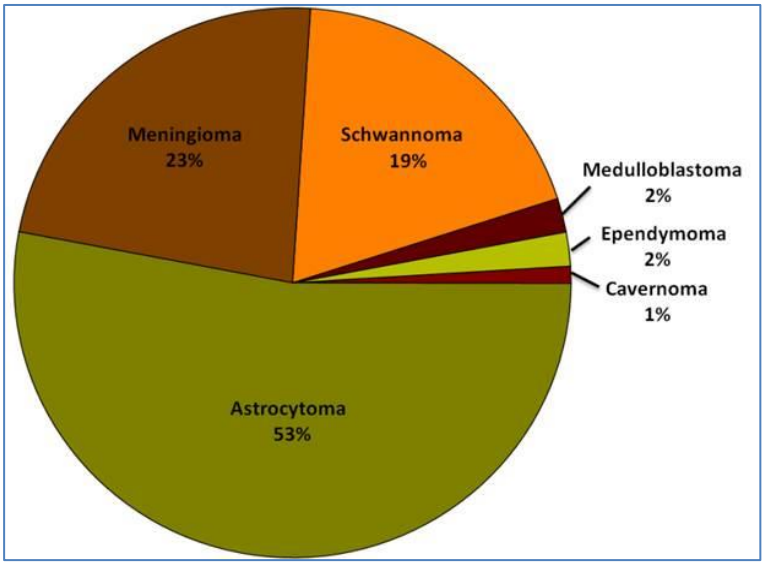

Figure 6. Statistical distribution of brain tumors [Deshpande et al. 2016]

The regular therapeutics regimen includes surgical resection followed by or along with radio and chemotherapy. Sometimes concomitant temozolomide (TMZ) can be employed as an adjuvant therapy (Hanif et al. 2017; Mrugala et al. 2014). The supportive care entails providing effective care of patients to overcome cognitive and neurological disorders manifested during regular therapeutics management (Hanif et al. 2017; Rampling et al. 2004). In spite of the enhancement in molecular understanding of GBM pathogenesis and several international efforts made by WHO and CBTRUS, GBM treatment still remains challenging with limited clinical outcomes.

Glioma risk factors

Several risk factors such as the genetic risk factor, ionizing and non ionizing risk factors and some chemical carcinogen have been considered as potential contributors to glioma(Pierce et al. 2018; Rice et al. 2016). However, except some genetic disorder such as Neurofibromatosis 1, Lynch syndrome, Li-Fraumeni syndrome and Ollier disease may risk for glioma (Rice et al. 2016); there is no clear evidences that other risk factors rendering to the cause of GBM.

Rationale of the present study

Cancer is a result of dynamic alteration in the deregulations of genes which either promote or inhibit proliferation by modulating several factors. These genetic alterations in tumor tissue are important in detecting the tumor stage, predicting prognosis and also for treatment (Furnari et al. 2007; Hanahan and Weinberg 2011).

With the confluence of advancement in cancer research, it is now clear that the tumor microenvironment plays an important role in tumor malignancy and chemoresistance (Bonavia et al. 2011; Ramirez et al. 2013). In 1931, Otto Heinrich Warburg described the concept of extracellular acidity through bioenergetics switching which is also widely known as 'the Warburg effect' (Warburg 1956). The tumor acidic microenvironment facilitates epithelial to mesenchymal transition (EMT) and NF-κB signaling that are pivotal for cancer progression, malignancy and drug resistance (Aggarwal 2004; Chen et al. 2016; Fearon and Vogelstein 1990; Gatenby and Gillies 2004; Marcucci et al. 2016; Ramirez et al. 2013). EMT is characterized by the loss of epithelial phenotype to gain of mesenchymal phenotype, that can be marked by epithelial markers such as E-cadherin, Zo-1 and γ-cadherin and mesenchymal markers such as vimentin, fibronectin and N-cadherin (Marcucci et al. 2016; Polyak and Weinberg 2009).

EMT recognition in glioma was recently defined and since in glioma glial cells acquire mesenchymal property, it is termed as glial to mesenchymal transition (GMT) or EMT-like process (Iser et al. 2017; Mahabir et al. 2014; Tso et al. 2006). EMT is induced by several signaling pathways, such as Wnt/β-catenin, phospho-inositide 3-kinase (PI3K), NF-κB and phospho-inositide 3-kinase-AKT-mTOR (mammalian target of rapamycin) (PI3K-AKT-mTOR) **(Figure 7)**. Activation of these signaling modulates EMT effectors such as Snail, Slug, ZEB1/ZEB2, STAT3, β-catenin, hypoxia-inducible factor 1α (HIF1α), TWIST etc. **(Figure 7)** (Marcucci et al. 2016; Mikheeva et al. 2010; Priester et al. 2013).

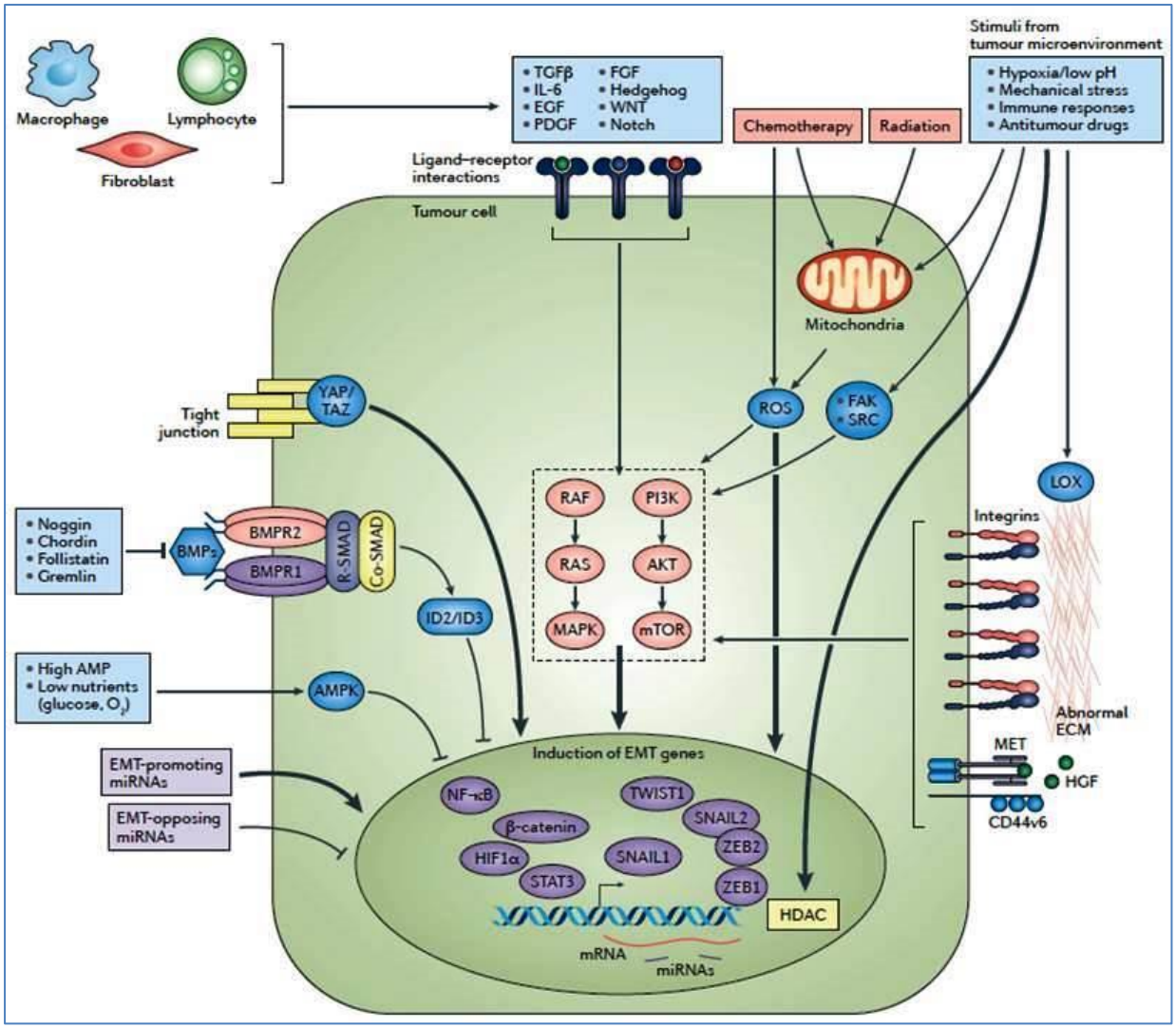

Figure 7. Induction of EMT signaling by various molecular and tumor microenvironment stimuli [Marcucci et al. 2016]

In addition to molecular stimuli, tumor microenvironment such as hypoxia and low pH also induce EMT (**Figure 7)** (Marcucci et al. 2014; Marcucci et al. 2016). The low extracellular pH of tumor cells is driven by the increased activity of pH regulators such as V-ATPase, Na^+/H^+Exchanger, and the carbonic anhydrase (Di Cristofori et al. 2015; Lee and Tannock 1998; Martinez-Zaguilan et al. 1999; Robertson et al. 2004). Tumor cells, on account of low extracellular pH, exhibits aggravated tumor malignancy and resistance to chemo and radiotherapy (Dhup et al. 2012; Hirschhaeuser et al. 2011).

The proton pump inhibitors (PPIs) are acid activated prodrugs globally used to suppress acid refluxes and gastric ulcers with minimal side effects (Fais 2010; Thomson et al. 2010). The first observation for the anticancer activities of PPIs was reported using gastric cancer model (Yeo et al. 2004). Since then several emerging studies have been demonstrated the antineoplastic and chemosensitizing efficacy of PPI (Canitano et al. 2016; De Milito et al. 2010; Ferrari et al. 2013; Zhang et al. 2014a).

Breast cancer metastasis suppressor gene 1 (*BRMS1*) is a noted metastasis suppressor gene (MSG) that can cease metastasis without affecting tumor growth (Welch et al. 2000). Although it has been more than a decade from the discovery of *BRMS1* to its recognition as a potential tumor regulator gene, its expression and clinical significance are discrepant (Bucciarelli et al. 2018; Kodura and Souchelnytskyi 2015; Yang et al. 2016; Zhang et al. 2006; Zhang et al. 2014b). Availability of limited information in astrocytoma (Mei et al. 2014) advocates the necessity of further investigations of BRMS1 in astrocytoma.

Even though EMT is recognized in glioma, more investigations are needed to delineate its translational relevancies. Emerging reports indicate the therapeutic significance of EMT by PPI - pantoprazole in gastric cancer and BRMS1 in lung carcinoma (Feng et al. 2016; Liu et al. 2015; Zhang et al. 2015), but nothing is known in context to astrocytoma malignancy and chemoresistance. In the present study, we have investigated the clinical significance of EMT like process in glioma and its therapeutic relevance through the chemical and molecular approaches.

To achieve our hypothesis we proposed two major objectives:

I. **Portrayal of epithelial to mesenchymal transition (EMT) like process in human glioma and its therapeutic relevance to rabeprazole**

   a) Clinical significance of EMT like process in glioma

   b) The therapeutic potential of EMT by rabeprazole to combat glioma growth and temozolomide resistance

II. **Molecular dissection of breast cancer metastasis suppressor 1 (BRMS1) in astrocytoma and its etiological connection with EMT**

   a) **Distinct expression and role of BRMS1 in *mut*P53 GBM**

   b) **Clinical and therapeutic relevance of BRMS1 to EMT in *mut*P53 GBM**

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Chapter 2

Portrayal of epithelial to mesenchymal transition (EMT) like process in human glioma and its therapeutic relevance to rabeprazole

Objective 1

- Clinical significance of epithelial to mesenchymal transition (EMT) like process in human glioma

Objective 2

- Therapeutic potential of EMT by rabeprazole to combat glioma growth and temozolomide resistance

Abstract

The acidic microenvironment of the tumor accelerates epithelial to mesenchymal transition (EMT) and NF-κB activation aggravating glioma malignancy and chemoresistance. Here, we investigated the clinical significance of EMT like process in human glioma and its therapeutic potential using rabeprazole in glioma *in-vitro* and *in-vivo*. The expression analysis of EMT associated proteins revealed the non-canonical expression of E-cadherin or N-cadherin and upregulation of GFAP, vimentin, and β-catenin in human glioma which may attribute to glioma malignancy and poor prognosis. Rabeprazole treatment attenuated glioma cell growth, cell migration and induced apoptosis *in-vitro* and *in-vivo*. Rabeprazole exposure suppressed EMT by repressing NF-κB signaling and/or inhibiting Akt/GSK3β phosphorylation. Further, the *in-vivo* study corroborates *in-vitro* results with augmented animal survival in the confluence of EMT inhibition. We also showed temozolomide sensitizing effect of rabeprazole on temozolomide resistant cell line. In conclusion, the two major finding of our study are: (i) we evidenced the clinical significance of EMT like process in glioma malignancy and poor prognosis and (ii) it's therapeutic relevance to rabeprazole treatment along with rabeprazole anticancer and chemosensitization efficacy; warrants rabeprazole repurposing for therapeutic interventions.

2.1 Introduction

Glioblastoma multiforme (GBM) is a highly invasive form of glioma that covers 55.4% of the malignant glioma with least (5.5%) five-year post diagnostic survival (Ostrom et al. 2016). The probable reason for low GBM survival is resistance against the currently available standard of care owing to its complex genetic and phenotypic heterogeneity making it globally one of the most noted malignancies (Beier et al. 2011; Furnari et al. 2007; Ramirez et al. 2013).

The acidic microenvironment of tumor facilitates epithelial to mesenchymal transition (EMT) and NF-ϰB signaling that are pivotal for cancer progression, malignancy and drug resistance (Aggarwal 2004; Chen et al. 2016; Fearon and Vogelstein 1990; Gatenby and Gillies 2004; Marcucci et al. 2016; Ramirez et al. 2013). EMT is characterized by the loss of epithelial phenotype to gain of mesenchymal phenotype, that can be marked by epithelial markers such as E-cadherin, Zo-1 and γ-cadherin and mesenchymal markers such as vimentin, fibronectin and N-cadherin (Marcucci et al. 2016; Polyak and Weinberg 2009). EMT recognition in glioma was recently defined and since glial cells acquire mesenchymal property, it is termed as glial to mesenchymal transition (GMT) or EMT-like process (Iser et al. 2017; Mahabir et al. 2014; Tso et al. 2006). EMT is induced by several signaling pathways, such as Wnt/β-catenin, phospho-inositide 3-kinase (PI3K), NF-ϰB and phospho-inositide 3-kinase-AKT-mTOR (mammalian target of rapamycin) (PI3K-AKT-mTOR) (Marcucci et al. 2014; Marcucci et al. 2016; Martinez-Zaguilan et al. 1996; Sahlgren et al. 2008). Activation of these signaling modulates EMT effectors such as Snail, Slug, ZEB1/ZEB2, STAT3, β-catenin, hypoxia-inducible factor 1α (HIF1α) and TWIST etc. (Marcucci et al. 2016; Mikheeva et al. 2010; Priester et al. 2013). In addition to molecular stimuli, tumor microenvironment such as hypoxia and low pH also induce EMT (Marcucci et al. 2014; Marcucci et al. 2016).

The low extracellular pH of tumor cells is driven by increased activity of pH regulators such as V-ATPase, Na^+/H^+Exchanger, and the carbonic anhydrase (Di Cristofori et al. 2015; Lee and Tannock 1998; Martinez-Zaguilan et al. 1999; Robertson et al. 2004). The proton pump inhibitors (PPIs) such as esomeprazole, lansoprazole, pantoprazole and rabeprazole are acid activated prodrugs globally used for stomach acidity and gastric ulcer with minimal adverse effects (Stedman and Barclay 2000; Thomson et al. 2010). Recent reports demonstrated EMT inhibition by pantoprazole in gastric cancer (Feng et al. 2016; Zhang et al. 2015).

Rabeprazole, a potent second generation PPI, can inactivate H^+/ K^+-ATPase very efficiently compared to other PPIs, without affecting healthy tissue, even at high concentrations (Besancon et al. 1997; Pantoflickova et al. 2003; Stedman and Barclay 2000). The anti-proliferative efficacy of rabeprazole in gastric cancer cells (Gu et al. 2014) indicates scope of utilizing its anticancer potency against glioma in association with EMT inhibition, as the low extracellular pH of a tumor cell is pivotal for invasive property of the tumor cells and EMT induction (Marcucci et al. 2014; Martinez-Zaguilan et al. 1996).

In the current study, we have assessed EMT like process in human glioma tissues using EMT proteins and correlated with various clinicopathological parameters and patient's survival. Further, we demonstrated the potential of rabeprazole as anti-glioma therapeutic using various cell-based assays in multiple malignant glioma cell lines at physiological and acidic conditions by suppressing EMT. The *in-vivo* study using C6 rat glioma model corroborates with *in-vitro* results and augments median survival age of rabeprazole treated animals. Furthermore, we also evidenced temozolomide (TMZ) sensitization by rabeprazole *in-vitro* using a TMZ resistant cell line.

2.2 Materials and methods

2.2.1 Patient specimens

Surgically resected glioma (n=44) and control (n=7) tissues were collected from the Krishna Institute of Medical Sciences (KIMS), Secunderabad, India. The study group comprised 44-glioma tissues (16 grade II, 12 grade III and 16 grade IV) classified according to WHO histopathological grading system (Louis et al. 2007) and 7 non tumor temporal epilepsy tissues were considered as controls. Written informed consent was obtained from all the study subjects. Institutional Ethics Committee (IEC), University of Hyderabad and KFRC-Ethics committee (KIMS) approved the use of human tissues for the experimental purpose.

2.2.2 Cell culture

Rat glioma cell lines C6 and human GBM cell lines U373, U87 and LN18 were procured from NCCS- Cell repository, Pune, India. T98 cell line was a kind gift from Dr. Ellora Sen (National Brain Research Center, Gurgaon, India). Cell lines included in this study were authenticated and free from mycoplasma contaminations. All cell lines were maintained in RPMI medium, supplemented with 10% fetal bovine serum and 100 IU/ml penicillin and 100µg/ml streptomycin at 37^0C in 5% CO_2 chamber. All experiments were performed in buffer medium pH-7.4 with sodium bicarbonate and unbuffered medium without sodium bicarbonate.

2.2.3 Chemicals and reagents

Rabeprazole was extracted from aciphex tablets, which were pulverized in to fine powder, diluted with water and extracted into dichloromethane. Over three water washings, the combined organic layer was dried over anhydrous Na_2SO_4 and concentrated under reduced pressure to get beige colored solid which was characterized by Mass, ^{1}H and ^{13}C NMR as rabeprazole.

2.2.4 Reagents

RPMI-1640 medium purchased from (Hi-Media), FBS (10270), anti-anti (15240-062) and sodium bicarbonate purchased (25080) from Gibco, MTT (M2128), Trypan blue (T-0776), Propidium iodide (P4170), DCFDA (D6883), RH-123 (R8004) were obtained from Sigma Aldrich. Cell lysis buffer (CST-9803) and All primary antibodies β-actin (#4970), apoptosis antibodies (#9950), EMT antibody (#9782) and secondary antibodies (#7074, #7076) were purchased from cell signaling technology (CST).

2.2.5 Cell proliferation/viability

Rabeprazole dose-dependent inhibition of cell proliferation was determined using MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] as reported previously (Du et al. 2004; Sareddy et al. 2012). Six thousand cells were seeded in each well of 96-well plates and kept in the CO_2 chamber for overnight growth. The medium was then replaced with fresh buffer or unbuffered medium and kept in the CO_2 chamber for 4 hours of incubation to acidify medium (Azzarito et al. 2015). Further, dose and time dependent treatment with rabeprazole and vehicle (0.1% DMSO) were given to the cells. Absorbance was measured at 570 nm using a multi-plate reader (Tecan Infinite-200). Cell death percentage was determined by trypan blue exclusion test after three doses (50 μM, 100 μM and 200 μM) of rabeprazole and the vehicle. The cell death percentage was calculated using the formula; the number of dead cells (stained cell)/ total number of cells (stained + unstained) x 100; upon 0.4% trypan blue staining.

2.2.6 Flow cytometry-PI, RH-123 and DCFDA staining

Briefly, C6 cells were trypsinized and seeded at a density of $1x10^5$ cells in six-well plates. After overnight growth, medium was replaced with fresh buffer and unbuffered medium and allowed for 4 hours incubation in the CO_2 chamber. Cells were treated with 50 μM and 100 μM of rabeprazole and 0.1% of the vehicle. After 24 hours of treatment, cells were harvested, fixed

with 70% of ethanol and stained with propidium iodide (PI) mixture (50µg/ml PI,1% triton-x and 50µg/ml RNase A) in dark for 20 min at room temperature (Du et al. 2004). For reactive oxygen species (ROS) estimation above mentioned rabeprazole doses were used with 20 µM H_2O_2 as a positive control. Three different concentrations of rabeprazole were used for RH-123 staining. After treatment, cells were trypsinized, washed with PBS and stained with 20 µM of DCFDA dye and RH-123 working solution (20nmol/l) for 30 min in dark at room temperature. Further, PI, DCFDA and RH-123 stained cells were processed by flow cytometry using FACS Caliber (BD Biosciences) and data was analyzed by FCS express 5 software.

2.2.7 Clonogenic assay

Clonogenic assay was performed by plating $1x10^3$ C6 cells in each well of six-well plates and kept in the CO_2 chamber for growth. After 24 hours of growth, the medium was replaced with fresh buffer and unbuffered medium and allowed for 4 hours incubation. After incubation, the cells were treated with three different doses (50 µM, 100 µM, 200 µM) of rabeprazole and vehicle (0.1%) for 24 hours. Then cells were allowed to grow for 7 days or until colony appears and stained with 0.1% crystal violet in 75% methanol. Colonies were counted as previously described (Du et al. 2004).

2.2.8 Wound healing assay

C6 cells ($1x10^5$) were seeded in a six wells plate and allowed to grow overnight in CO_2 chamber and after overnight growth, a vertical and horizontal wound was made. Wound areas were washed with PBS and fresh buffer and unbuffered medium were added and kept in the CO_2 chamber for 4 hours of incubation. After incubation, cells were treated with three different doses (25 µM, 50 µM, and 100 µM) of rabeprazole and vehicle (0.1%) for 24 hours. Photographs were taken from the same position at different time intervals.

The wound-healing percentage was calculated by the ratio of the healing area at each time point to the wound area at zero hour. Wound-healing coverage was determined from two different fields and the results were expressed as mean ± SEM.

2.2.9 Transwell migration assays

Cell migration was checked by transwell inserts with polycarbonate membrane (8-micron pore size). Upon rabeprazole treatment, the cells were seeded in transwell inserts in serum free media and kept in a CO_2 chamber for 12 hours incubation. FBS was used as a chemo attractant. After 12 hours of growth, inserts were carefully removed and washed with PBS twice. Traversed cells were fixed in methanol and stained with 1% crystal violet. To analyze the results, six individual fields of traversed cells of the membrane were photographed and counted.

2.2.10 Western blots

Tissues and cells were lysed in lysis buffer containing protease inhibitor cocktail as described earlier (Du et al. 2004; Sareddy et al. 2012). Proteins were separated by SDS-PAGE and transferred onto nitrocellulose membrane. After one hour of blocking, the membranes were probed with specific antibodies at 4°C overnight, followed by one hour incubation with horseradish peroxidase-conjugated secondary antibody at room temperature. Immunoreactivity was visualized by Bio-rad Chemidoc imaging system.

2.2.11 Animals/stereotaxic glioma model

Male wistar rats of 4-5 months with 180-220 grams of weight were obtained from National Institute of Nutrition (NIN) Hyderabad, India. Before stereotaxic implantation, all animals were housed for minimum 15 days in an animal house facility of the University of Hyderabad. Stereotaxic implantation was performed as described earlier (Smilowitz et al. 2007).

Briefly, C6 glioma cell suspensions ($2x10^5$ cells in 10 µl) were injected in the anesthetized rat brain (striatum region) through a drilled burr hole using a Hamilton syringe and the cavity

was filled with dental cement. Rats were closely monitored daily for tumor symptoms and after 6 days of cells implantation, rats were divided into two groups of six rats each. Group (a) received five doses of vehicle (5% DMSO) and group (b) received five doses of rabeprazole (40mg/kg of body weight) at the interval of 48 hours through the tail vein. Animal experiments were performed in accordance with Institutional Animal Ethics Committee (IAEC), University of Hyderabad.

2.2.12 Histological analysis

For the evaluation of antitumor efficacy of rabeprazole, we removed rat brain after perfusion (Gage et al. 2012) and fixed in 4% paraformaldehyde for at least 48 hours. Then 5-10 micron brain tissue sections on a silane coated glass slide were prepared by cryotome, followed by hematoxylin and eosin (H&E) staining using routine lab protocol. For immunohistochemistry (IHC)/immunofluorescence (IF) staining, tissue sections were dewaxed, rehydrated and stained by previously described method (Sareddy et al. 2009). Tissue sections were thoroughly washed with phosphate buffered solution (PBS) in each step, visualized with 3,3'-diaminobenzidine tetra hydrochloride, and counterstained with hematoxylin in case of IHC. The quantification of positively stained/unstained cells was performed by counting cells of minimum six random fields of a tissue section. At least three brain sections were separately stained in duplicates.

2.2.13 Statistical Analysis

All data were analyzed through statistical software SigmaPlot 11.0 and GraphPad Prism 5. The results are expressed as mean ± SEM, the P value is calculated using One-way ANOVA for multiple groups or student t-test for two groups as noted, $p \leq 0.05$ accepted as significant, (*) indicates $p \leq 0.05$, (**) = $p \leq 0.02$ and (***) = $p \leq 0.001$. Survival data were analyzed by using Kaplan Meier survival curve and compared by Log-rank (Mantel-Cox) test. All experiments were performed in triplicates and repeated thrice otherwise mentioned.

2.3 Results

2.3.1 EMT like process associated with glioma malignancy and poor prognosis

We checked EMT profile using EMT associated proteins vimentin, β-catenin, E-cadherin, N-cadherin and GFAP in different grades of glioma (n=44) and control tissues (n=7). Western blotting results revealed increased expression of vimentin, β-catenin and GFAP in different grades of glioma and negative or weak expression of E-cadherin and N-cadherin in glioma grades compared to the control tissues indicating EMT like process in glioma **(Figure 1A)** and **(Supplementary Figure S1 A)**. The IHC staining showed significantly increased cytosolic vimentin (p=0.001) and nuclear β-catenin (p=0.01) levels in glioma grades as compared to the control tissues **(Figure 1B-E)**. The cytosolic vimentin was also confirmed with IF **(Supplementary Figure S1 B, C)**. Of the total cohort, 86.36% tissues showed negative expression of E-cadherin while positive expression of GFAP, vimentin and β-catenin was observed in 88.36%, 65.90% and 72.72% of tissues respectively **(Table 1 and Supplementary Table S1)**. Further, the clinicopathological analysis revealed that E-cadherin, GFAP, vimentin and β-catenin expression is associated with histological grading and independent of age and sex **(Table 1)**. The correlation study demonstrated a negative association of E-cadherin and positive associations of GFAP and β-catenin with vimentin **(Supplementary Figure S2 A-C and Supplementary Table S2)**. Log-rank (Mantel-Cox) survival graph illustrated patient's poor survival with negative E-cadherin (p=0.7343) expression **(Figure 1F)** while GFAP (p=0.2831), vimentin (*p=0.0091) and β-catenin (*p=0.0175) positive expressions **(Figure 1G-I)** were associated with poor prognosis and reduced median survival age, suggesting that EMT targeting can improve tumor malignancy and patient's survival.

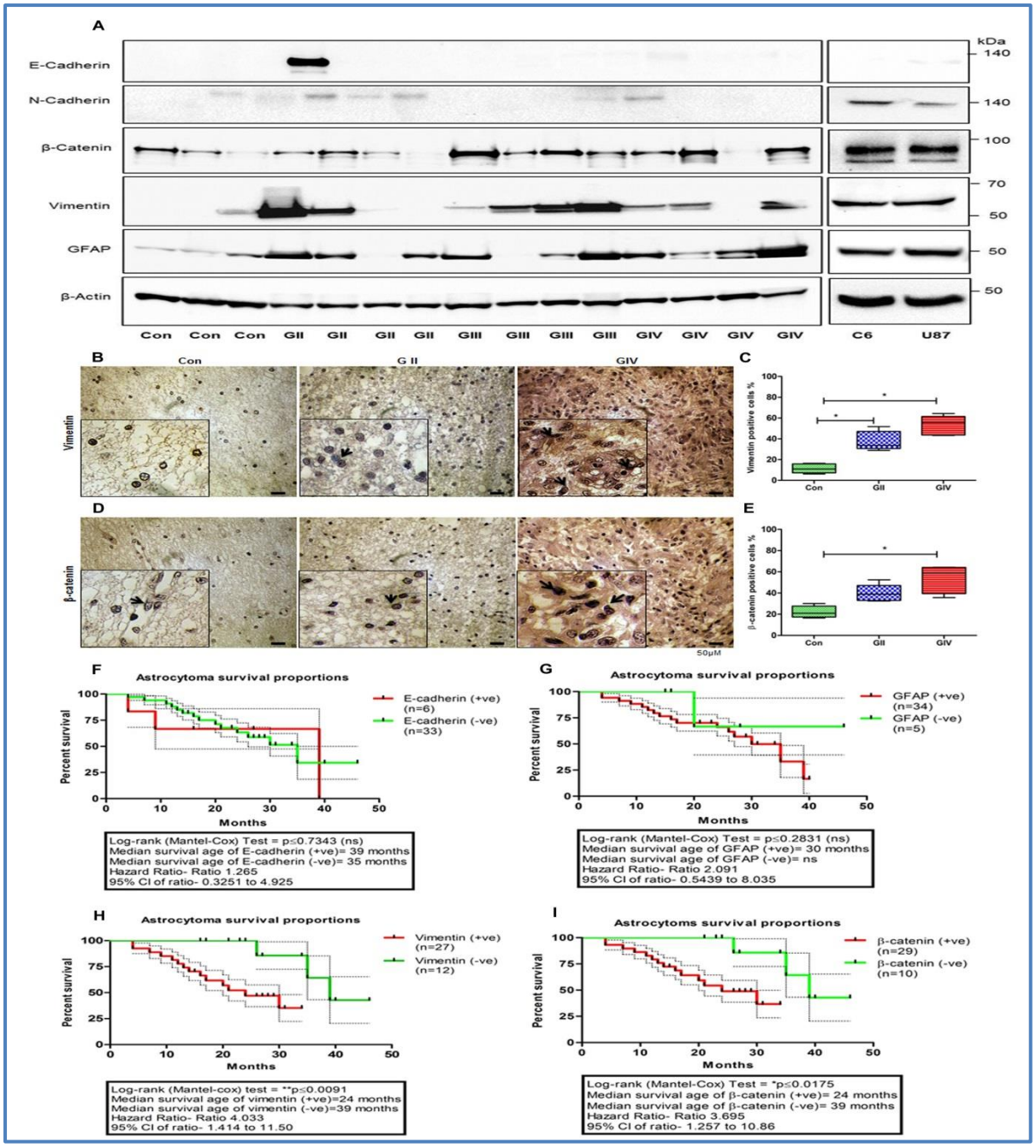

Figure 1. Increase EMT proteins in glioma grades associated with poor patients' survival. **(A)** Representative western blotting images depict a weak or null expression of N-cadherin and E-cadherin. The upregulated expression of GFAP, vimentin and β-catenin in different grades of glioma and cell lines as compared to the control tissues; β-actin was used as an internal control. **(B, D)** IHC staining with anti vimentin and anti β-catenin shows increase vimentin and β-catenin in glioma grades as compared to the control tissues. **(C, E)** IHC quantification reveals the significant increase of vimentin (p=0.001) and β-catenin (p=0.01) in GIV in comparison to the low grade and control tissue. **(F-I)** The Kaplan-Meier graph discloses EMT proteins association with glioma malignancy that inflicts poor prognosis, Log-rank (Mantel-Cox) test indicates **(F)** E-cadherin negativity associated with poor survival (p=0.010). **(G-I)** Increased GFAP (p=0.001), vimentin (p=0.012) and β-catenin (p=0.011) expression patterns reveals patients poor survival.

Table 1. The expression pattern of EMT associated proteins and follow up details with various clinicopathological features of astrocytoma patients

S.No.	Tumor grade	Age/Sex	E-Cadherin	GFAP	Vimentin	β-catenin	Follow-up time (month)
			-ve or +ve	-ve or +ve	-ve or +ve	-ve or +ve	
1	Con	20/M	-ve	+ve	-ve	-ve	26 L
2	Con	26/M	-ve	+ve	-ve	+ve	25 L
3	Con	42/F	-ve	+ve	-ve	+ve	46 L
4	Con	9/M	+ve	+ve	-ve	-ve	35 L
5	Con	24/F	-ve	-ve	-ve	+ve	28 L
6	Con	38/M	-ve	+ve	-ve	-ve	35 L
7	Con	52/F	-ve	+ve	-ve	+ve	40 L
8	GII	35/F	-ve	+ve	+ve	+ve	16 L
9	GII	36/M	-ve	-ve	+ve	+ve	16 L
10	GII	46/M	-ve	+ve	-ve	+ve	17 L
11	GII	35/F	-ve	+ve	-ve	+ve	16 L
12	GII	52/F	-ve	+ve	-ve	-ve	26 D
13	GII	25/F	+ve	+ve	+ve	+ve	04 D
14	GII	10/M	-ve	+ve	-ve	-ve	24 L
15	GII	47/M	+ve	+ve	+ve	-ve	27 L
16	GII	56/M	-ve	+ve	+ve	+ve	NA
17	GII	51/F	-ve	+ve	+ve	+ve	30 D
18	GII	43/M	-ve	-ve	+ve	+ve	28 L
19	GII	56/M	-ve	+ve	-ve	-ve	34 L
20	GII	44/F	-ve	+ve	+ve	+ve	21 D
21	GII	37/M	-ve	+ve	+ve	+ve	13 D
22	GII	36/M	+ve	+ve	-ve	+ve	21 L
23	GII	21/F	-ve	+ve	+ve	+ve	29 L
24	GIII	16/M	-ve	+ve	-ve	-ve	40 L
25	GIII	50/M	-ve	+ve	-ve	-ve	NA
26	GIII	48/M	-ve	-ve	+ve	+ve	15 L
27	GIII	52/M	-ve	+ve	+ve	+ve	04 D
28	GIII	42/M	-ve	+ve	+ve	+ve	31 L
29	GIII	12/M	-ve	+ve	+ve	+ve	26 L
30	GIII	53/M	-ve	+ve	-ve	-ve	35 D
31	GIII	31/M	-ve	+ve	-ve	-ve	NA
32	GIII	41/F	-ve	+ve	+ve	+ve	30 L
33	GIII	51/F	-ve	+ve	+ve	+ve	29 L
34	GIII	30/M	+ve	+ve	+ve	+ve	16 L
35	GIII	14/F	-ve	+ve	-ve	-ve	23 L
36	GIV	52/F	-ve	+ve	+ve	+ve	17 D
37	GIV	71/M	-ve	+ve	+ve	+ve	14 D
38	GIV	59/M	-ve	+ve	+ve	-ve	21 L
39	GIV	63/F	-ve	+ve	+ve	+ve	11 D
40	GIV	45/F	-ve	+ve	+ve	+ve	24 D
41	GIV	70/M	-ve	+ve	+ve	+ve	12 D
42	GIV	44/M	+ve	+ve	-ve	-ve	39 D
43	GIV	41/F	-ve	+ve	+ve	+ve	07 D
44	GIV	58/M	-ve	+ve	+ve	+ve	NA
45	GIV	52/M	-ve	+ve	-ve	+ve	NA
46	GIV	17/F	+ve	+ve	+ve	+ve	09 D
47	GIV	49/M	-ve	-ve	-ve	-ve	46 L
48	GIV	42/F	-ve	-ve	+ve	+ve	20 D
49	GIV	56/M	-ve	+ve	+ve	+ve	16 D
50	GIV	28/M	-ve	+ve	-ve	+ve	27 L
51	GIV	63/M	-ve	+ve	+ve	+ve	34 L

 # +ve and –ve represent presence and absence of protein respectively, L: Live, D: Dead

2.3.2 Rabeprazole attenuated glioma cell growth and induced cell death *in-vitro*

To study the anti-cancer efficacy of rabeprazole on glioma cells *in-vitro*, we first determined rabeprazole effect on C6 cell using MTT-assay. As shown in **Figure 2A**, C6 cells were treated with ten different concentrations and vehicle (0.1%) for 24 hours in both medium. The half-inhibitory concentration (IC_{50}) was observed at 100 μM in buffer medium ~50 μM in unbuffered medium **(Figure 2A)**. The phase contrast and fluorescent microscopy indicated altered C6 cells morphology in terms of cell size, shape and nuclear fragmentation after rabeprazole treatment at 100 μM in both the conditions **(Figure 2B, C)**.

Further, we used three human glioblastoma cell lines T98, U87, U373 in addition to C6 cells, to assess the cell growth and cell death efficacy upon rabeprazole treatment. Among all four cell lines, C6 cells exhibited 50% inhibition in growth rate at 100 μM while T98, U87, U373 showed IC_{50} at 200 μM of rabeprazole concentration in a buffered medium upon 24 hours treatment **(Figure 2D-G)**. In unbuffered condition, C6 and T98 cells showed less than 50% inhibition in growth rate at 50 μM concentration of rabeprazole **(Figure 2D, E**). Cell death experiment results demonstrated ~50% cell death in C6 and T98 cells in buffered medium at 100 μM concentration while more than 50% cell death was observed at 50 μM of rabeprazole dose in the unbuffered medium after 24 hours treatment **(Figure 3A, B)**. U373 and U87 cells display 50% cell death at 200 μM in buffer medium while in unbuffered medium more than 50% cell death was observed at 100 μM after 24 hours of treatment **(Figure 3C, D)**. These results enhanced upon 48 hours of rabeprazole treatment indicating the significance of buffered and unbuffered medium.

2.3.3 Rabeprazole affected cell cycle phases and induced mitochondrial apoptosis

To identify the effect of rabeprazole on cell cycle, we performed PI stained DNA profiling in different cyclic phases such as G0/G1, S and G2/M through FACS analysis.

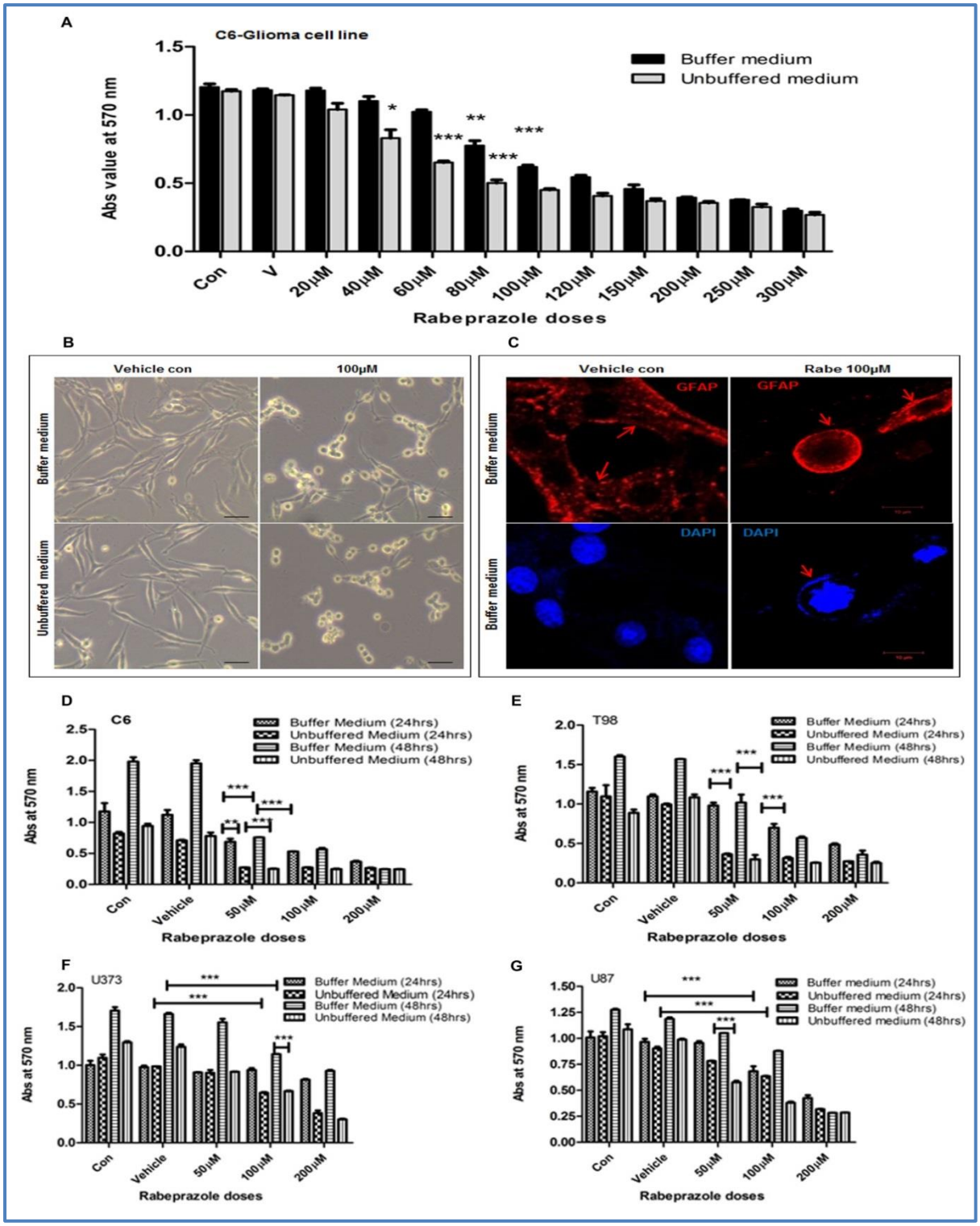

Figure 2. Rabeprazole inhibits rate of cell proliferation alter cell morphology. (A)C6 cells were treated with ten different doses of rabeprazole in both buffered and unbuffered media. The graph shows fifty percent inhibition (IC_{50}) at 100 µM rabeprazole dose in the buffered medium while in the unbuffered medium IC_{50} value observes at 50 µM after 24 hours of exposure. **(B and C)** The phase contrast and IF images show alter cell and nuclear morphology in terms of cell size shape and granularity upon rabeprazole exposure as compared to the vehicle control. **(D)** C6 graph represents 50% inhibition at 100 µM while **(E-G)** T98, U87 and U373 shows 50% inhibition at 200 µM of rabeprazole

concentration in the buffered medium upon 24 hours treatment. In the unbuffered medium, **(D, E)** C6 and T98 cells show <50% of inhibition at 50 μM concentrations of rabeprazole upon 24 hours treatment indicate enhanced efficacy of rabeprazole in acidic conditions. At least three independent experiments were performed in triplicates; bars indicate SEM. (*) indicate $p \leq 0.05$, (**) = $p \leq 0.02$, (***) = $p \leq 0.001$.

Rabeprazole treatment increased G1 phase percentage with increasing concentrations in buffered and unbuffered medium indicating that rabeprazole exposure arrested cell cycle at G0/G1 phase **(Figure 3E)** suggesting its role in apoptotic cell death. The western blots showed increased active poly (ADP-ribose) polymerase (PARP) and caspase3 levels together with altered pro and anti apoptotic mitochondrial proteins in rabeprazole treated cells **(Figure 3F)** further conferring apoptosis. Next, we evaluated ROS levels by DCFDA dye and mitochondrial integrity by rhodamine 123 (RH-123) staining in both medium. The results indicated significant induction of ROS levels even more than positive control (H_2O_2) at 100 μM of rabeprazole concentration in unbuffered condition compared to the buffered medium **(Figure 3G)**. Reduced RH-123 fluorescence intensity or histogram shifting towards y-axis upon rabeprazole treatment confirmed mitochondrial depolarization **(Figure 3H)**. The terminal deoxynucleotidyl transferase (TdT) dUTP nick-end labeling (TUNEL) assay demonstrated high FITC staining (TUNEL positive cells) at 100 μM of rabeprazole dose as compared to the vehicle (0.1%) in both the medium **(Figure 3I)**.

Furthermore, we also showed increased cytochrome-c protein in cytosolic fraction of rabeprazole treated cells in both the conditions **(Figure 3J)**. These results clearly indicated that the rabeprazole treatment induced apoptotic cell death in a dose-dependent manner with more increased response in an acidic environment. β-actin was used as a loading control.

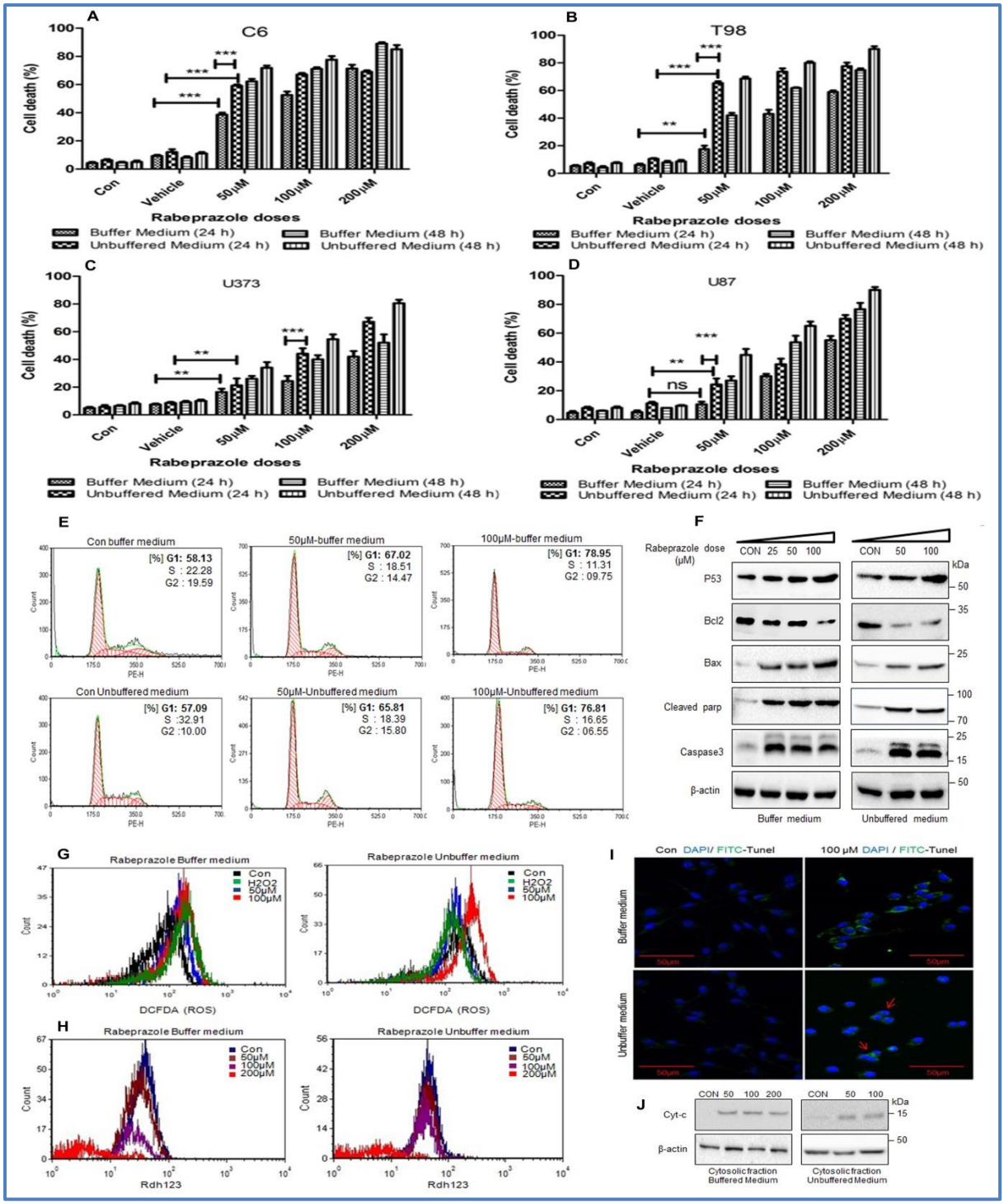

Figure 3. Rabeprazole induces mitochondrial mediated apoptotic cell death. (A-D) Rabeprazole treatment induces cell death of C6, T98, U373 and U87 cells in both the conditions with enhanced effects in acidic condition. **(E)** DNA flow cytometric study of C6 cells, showed dose-dependent G0/G1 phase arrest at 50 μM and 100 μM of rabeprazole dose in both medium, compared to the vehicle. **(F)** Western blots indicate increased active PARP, caspase-3 and pro-apoptotic proteins upon rabeprazole treatment in both the medium. β-actin was used as a loading control. **(G)** ROS accumulation in both conditions shown by DCFDA shift compared to control. The histogram represents vehicle control, positive control (H_2O_2), and 50 μM or 100 μM rabeprazole doses.

(H) Mitochondrial integrity by RH-123 staining; histogram shows fluorescence shifting toward the y-axis compared to control upon rabeprazole treatment. Black, green, blue and red line represents vehicle control, positive control, 50 μM, and 100 μM rabeprazole doses respectively. **(I)** Increased FITC-tunel staining at 100 μM of rabeprazole concentration in comparison to the control in both the conditions. **(J)** Cytochrome-c release in cytosolic fraction upon rabeprazole treatment.

2.3.4 Rabeprazole efficiently reduced colony formation and cell migration by targeting EMT through AKT/GSK-3β/β-catenin signaling inhibition

To determine the effect of rabeprazole on cell colonization and cell migration ability, we performed clonogenic, wound healing and transwell migration assay. Clonogenic assay revealed dose dependent colony inhibition in both the conditions **(Figure 4A)**, more than 50% reduction in colonies count was observed at 200 μM dose of rabeprazole in buffered and 100 μM in unbuffered conditions **(Figure 4B)**. In fact, extremely low numbers of colonies were detected in unbuffered medium at 200 μM concentration in comparison to the vehicle confirming its increased efficacy in acidic condition **(Figure 4A, B)**. The wound healing assay results showed a significant reduction in wound closure efficiency of C6 glioma cells at 50 μM in buffered medium and 25 μM in unbuffered medium compared to the vehicle **(Figure 4C, D)**. Compromised cell migration efficiency upon rabeprazole exposure was also verified by transwell migration assays, which showed significantly reduced cell migration efficiency at 50 μM rabeprazole dose in both the conditions **(Figure 4E, F)**. Simultaneously, we were also interested in checking the effect of rabeprazole on EMT associated proteins, the western blots revealed attenuated vimentin, β-catenin, NF-κB-P65 and STAT3 expression upon rabeprazole treatment in both the medium **(Supplementary Figure S3 A)**. Immunofluorescence (IF) images and its quantification indicated significantly low vimentin expression at 100 μM of rabeprazole dose in both the medium **(Supplementary Figure S3 B, C),** confirming anti EMT efficacy of rabeprazole.

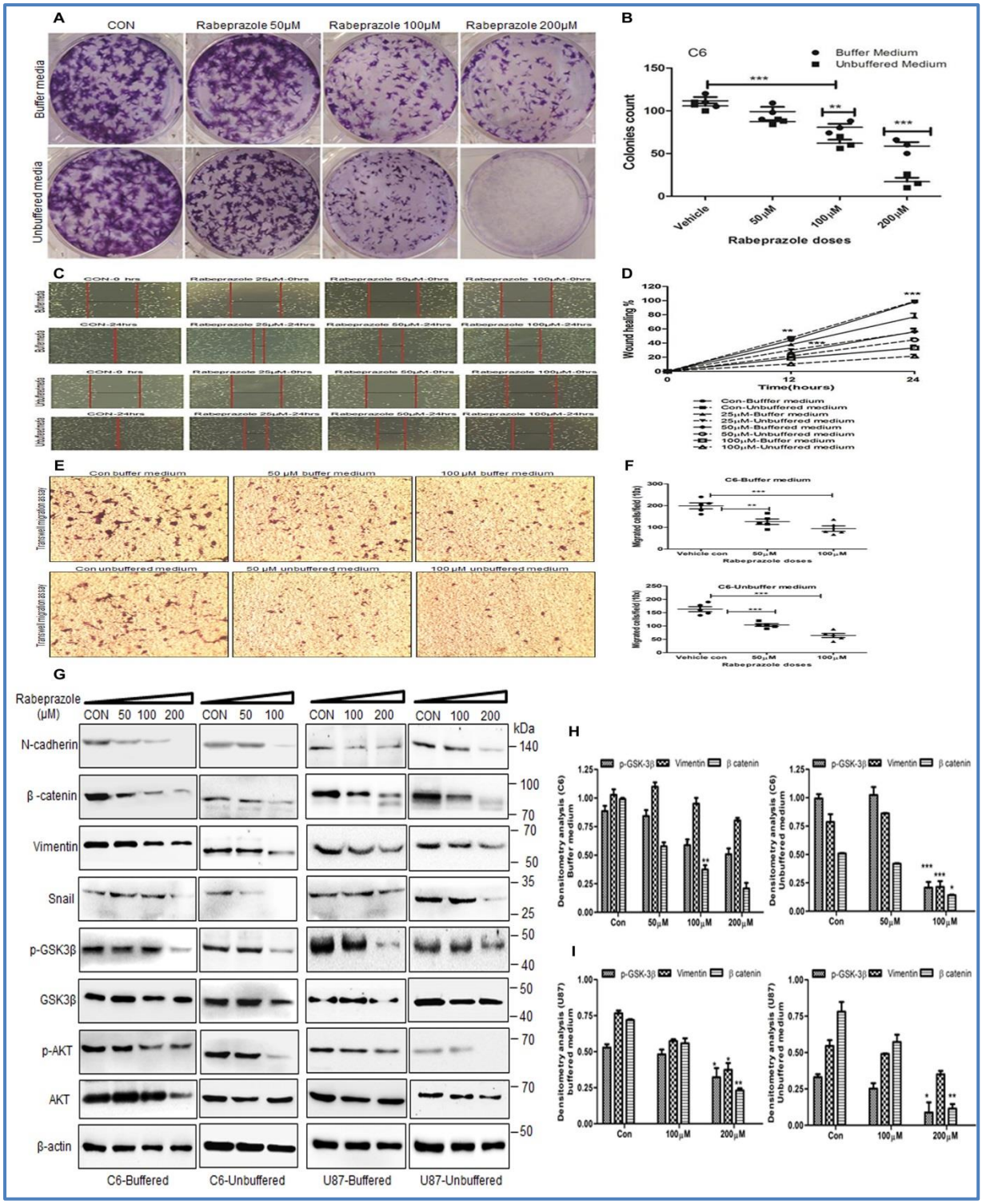

Figure 4. Rabeprazole reduces colony formation and cell migration by targeting EMT through Akt/Gsk-3β/ β-catenin signaling inhibition. (A) Clonogenic assay reveals a dose-dependent reduction in colony forming efficiency upon rabeprazole treatment. **(B)** The graph illustrates more than 50% colonies reduction at 200 μM and 100 μM in buffered and unbuffered medium respectively. **(C)** The images are the depictions of the wound-healing assay taken at two different time points (0 and 24 hours) after scratches formation. **(D)** The graph showed significant inhibition of wound healing percentage at a concentration of 50 μM in buffer medium while in unbuffered medium significant inhibition was observed at the concentration of 25 μM.

(E) Representative images of transwell migration assay showed restricted cell migration upon rabeprazole. **(F)** The graph indicates significant inhibition at 50 μM rabeprazole dose in both the conditions. **(G-I)** Western blots and its densitometry analysis upon rabeprazole exposure demonstrates significant inhibition of pAKT and pGsk-3β together with snail, β-catenin and EMT biomarkers (vimentin and N-cadherin) in C6 and U87 cell lines in both conditions. β-actin was used as an internal control.

Further, to identify the concomitant signaling we performed western blots of rabeprazole treated cells. Results revealed that rabeprazole efficiently inhibited EMT as depicted by decreased expression level of EMT proteins such as N-cadherin, vimentin, β-catenin and snail **(Figure 4G)**. Rabeprazole treatment also inhibited AKT and GSK-3β phosphorylation in a dose dependent manner in C6 and U87 cell lines in both conditions **(Figure 4G)** suggesting EMT repression through AKT/GSK-3β/β-catenin signaling. Densitometry analysis demonstrated significant inhibition of p-GSK-3β, vimentin and β-catenin in C6 and U87 cell lines in both the medium at 100 μM and 200 μM rabeprazole dose respectively **(Figure 4H, I)**.

2.3.5 Rabeprazole augmented median survival of glioma rat with reduced tumor growth in confluence of EMT inhibition.

To substantiate *in-vitro* evidences we performed *in-vivo* study using C6 stereotaxic rat glioma model. The morphological observation after five doses of rabeprazole (40mg/kg of body weight) revealed better physiology and cognition compared to the vehicle (5% DMSO) treated glioma rats **(Figure 5A)**. The Log-rank (Mantel-Cox) survival curves showed a significant difference ($p= 0.037$) between vehicle and rabeprazole treated glioma rats with increased median survival of 28.5 days for rabeprazole and 15 days for vehicle treated glioma rats **(Figure 5B)**. In fact, 3 out of 6 rabeprazole infused rats survived long-term up to 30 days; whereas, 3 out of 6 vehicle treated rats died before 15 days and only one rat survived up to 27 days of defined survival period (30 days) **(Figure 5B)**.

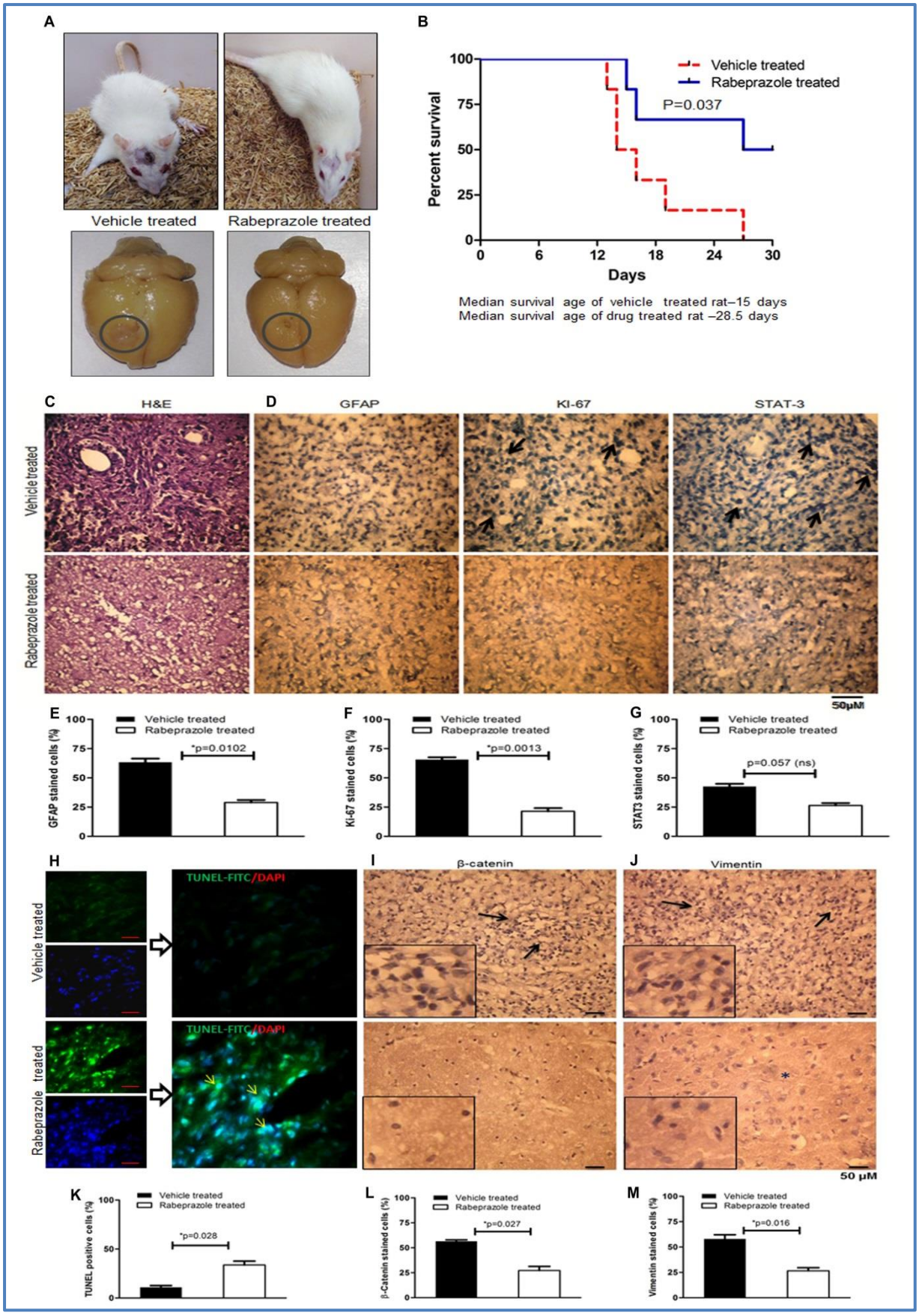
A
Vehicle treated
Rabeprazole treated
B
Vehicle treated
Rabeprazole treated
P=0.037
Percent survival
Days
Median survival age of vehicle treated rat–15 days
Median survival age of drug treated rat –28.5 days
C
H&E
D
GFAP
KI-67
STAT-3
Vehicle treated
Rabeprazole treated
50μM
E
GFAP stained cells (%)
*p=0.0102
F
Ki-67 stained cells (%)
*p=0.0013
G
STAT3 stained cells (%)
p=0.057 (ns)
H
TUNEL-FITC/DAPI
I
β-catenin
J
Vimentin
50 μM
K
TUNEL positive cells (%)
*p=0.028
L
β-Catenin stained cells (%)
*p=0.027
M
Vimentin stained cells (%)
*p=0.016

Figure 5. Rabeprazole improves animal survival with restricted tumor growth by targeting EMT like process. (A) Morphological images show restricted tumor growth in rabeprazole treated glioma rats (n=6) as compared to the vehicle treated glioma rats (n=6). **(B)** Kaplan-Meier survival curves depict significant difference p= 0.037 between rabeprazole and vehicle treated group with increased median age. **(C, D)** H&E and IHC staining show less tumor cells in rabeprazole treated rat as compared to the vehicle treated. **(D-G)** IHC images and its graphical quantification reveal a significant inhibition of GFAP (p=0.010), Ki-67 (p=0.001), and STAT3 (p=0.057) upon rabeprazole treatment as compared to the vehicle. **(H, K)** Tunel staining and its quantified graph present strong staining (p=0.028) in rabeprazole treated glioma rat as compared to the control rat indicating apoptotic cell death. **(I, J)** IHC images stained with anti β-catenin and anti vimentin show weak staining in rabeprazole treated glioma rats as compared to the vehicle treated glioma rats. **(L, M)** Graphs reveal significant inhibition of β-catenin (p=0.027) and vimentin (p=0.016) upon rabeprazole treatment. Minimum six random fields were selected for the quantification of an IHC stained tissue section.

The histopathological studies by H&E staining revealed restricted tumor growth in rabeprazole treated rats in comparison to the vehicle treated rats **(Figure 5C)**. IHC staining showed significantly weak GFAP (*p=0.010) and Ki-67 (*p=0.001) staining in rabeprazole treated rats compared to the vehicle treated glioma rat **(Figure 5D, E, F)** indicating reduced tumor cell growth, which are in agreement with the *in-vitro* findings. STAT3 a master regulator of EMT pathways and linked with cancer malignancy (Priester et al. 2013) was also found weakly stained in rabeprazole treated glioma rats compared to the vehicle treated glioma rats **(Figure 5D, G)** coincided *in-vitro* results. TUNEL staining showed strong positivity in rabeprazole treated glioma rats compared to the vehicle treated glioma rats **(Figure 5H)**. Quantification analysis illustrated significant difference in TUNEL staining between rabeprazole and vehicle treated glioma rats **(Figure 5K)** affirming apoptosis induction upon rabeprazole treatment. To evidence the EMT inhibition in *in-vivo* we performed IHC using anti vimentin and anti β-catenin, the IHC results showed weak β-catenin and vimentin staining in rabeprazole treated glioma rats as compared to the vehicle treated glioma rats **(Figure 5I, J)**.

Quantification assessment clearly showed significantly less positive staining of β-catenin (*p=0.027) and vimentin (*p=0.016) in rabeprazole treated glioma rats as compared to the vehicle treated glioma rats **(Figure 5L, M)**.

2.3.6 Rabeprazole attenuated TNF-α induced EMT like process by attenuating NF-ϰB signaling *in-vitro*

Further, to support the EMT repressing potency of rabeprazole we exposed TNF-α (20ng/ml) before rabeprazole treatment to stimulate EMT like process on C6 and U87 glioma cell lines. Western blots of TNF-α (20ng/ml) treated cell lines showed increased vimentin and β-catenin expression as compared to the control, which was further inhibited efficiently by rabeprazole in both conditions **(Figure 6A)**. Quantitative assessment of western blots by densitometry **(Figure 6B)** and IF imaging **(Figure 6C, D)** evidenced significant inhibition of vimentin at 100 μM of rabeprazole concentration even upon EMT induction by TNF-α. The role of NF-ϰB signaling in TNF-α induced EMT pathway has been reported earlier in other cancer (Li et al. 2012). We have also demonstrated the NF-ϰB-P65 activation and IKBα phosphorylation upon TNF-α (20ng/ml) exposure and their inhibition by rabeprazole doses in both the cell lines **(Figure 6E)**. NF-ϰB-P65 activation was justified by increasing p-IKBα and reducing total IKBα expression levels respectively **(Figure 6E, F)**. The IF images using C6 cells, showed attenuated nuclear translocation of NF-ϰB-P65 upon rabeprazole treatment as compared to the control **(Figure 6G)**. We also affirmed NF-ϰB suppression by NF-ϰB luciferase reporter assays that showed significant inhibition of NF-ϰB gene upon rabeprazole exposure in dose dependent manner **(Figure 6H)**.

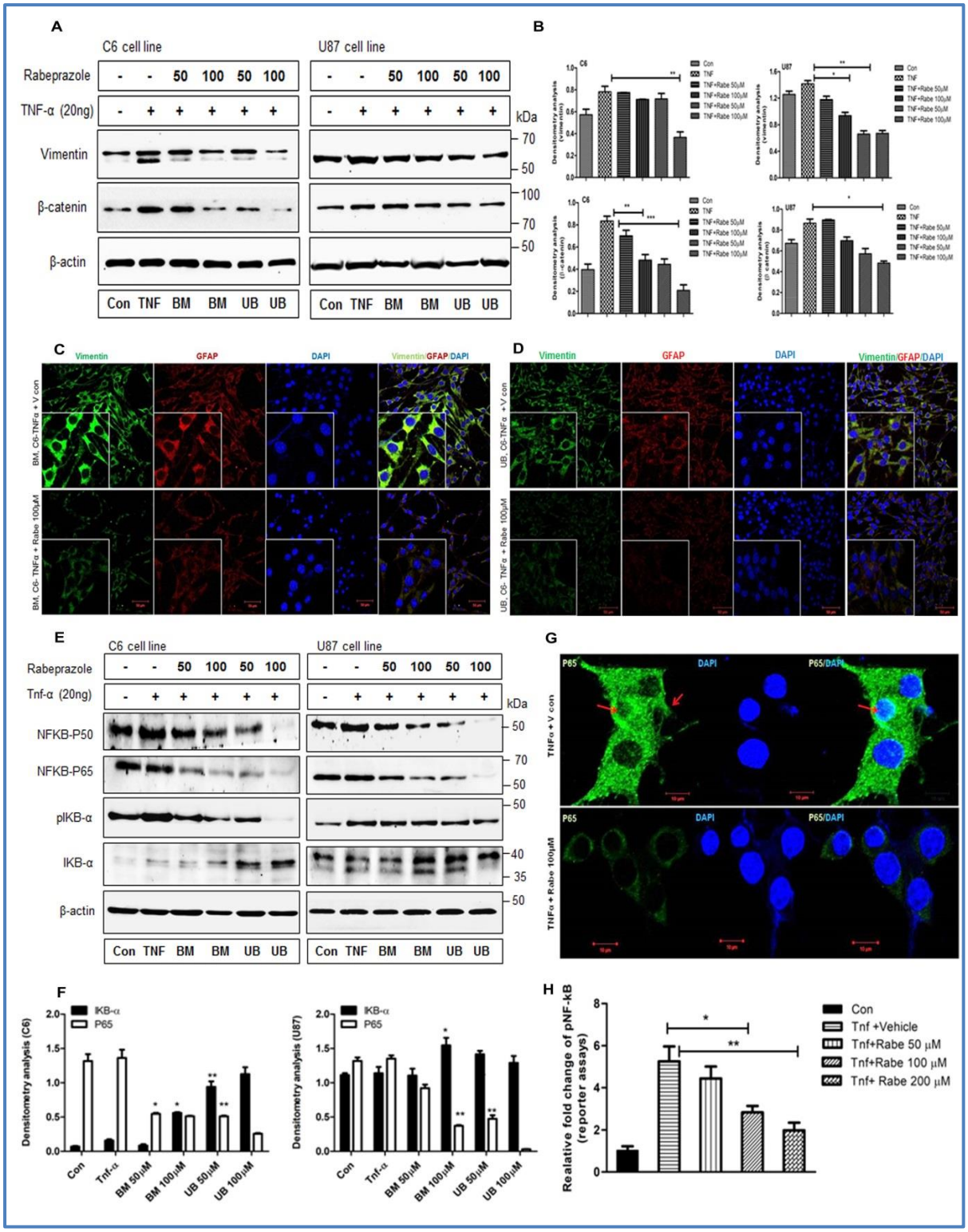

Figure 6. Rabeprazole suppresses TNF-α induced EMT by targeting NF-*κ*B signaling *in-vitro*. **(A)** Western blots of TNF-α (20ng/ml) treated cell lines illustrates increased vimentin and β-catenin expression compared to the control, and further suppressed by rabeprazole in C6 and U87 cells in both conditions. **(B)** Densitometry analysis shows significant inhibition of vimentin and β-catenin at 100 μM of rabeprazole concentration in both the cell lines. **(C, D)** The IF images also evidence the significant inhibition of vimentin at 100 μM of rabeprazole concentration upon EMT induction.

(E) Western blots demonstrate increased NF-ϰB-P50, NF-ϰB-P65 and p-IKBα proteins upon TNF-α (20ng/ml) exposure in both cell lines and further inhibited by rabeprazole doses. **(F)** NF-ϰB-P65 activation was justified by the densitometry graph showing reverse expression of NF-ϰB-P65 and total IKBα. **(G)** The IF images show attenuated nuclear translocation of NF-ϰB-P65 upon rabeprazole treatment. **(H)** NF-ϰB luciferase reporter assays demonstrate significant inhibition of NF-ϰB gene upon rabeprazole exposure in dose dependent manner.

2.3.7 TMZ sensitization by rabeprazole on TMZ resistant LN18 cell line

Previous studies have shown the chemosensitizing ability of PPI (Ferrari et al. 2013; Zhang et al. 2015). In order to evaluate the chemosensitizing efficiency of rabeprazole we used TMZ resistance LN18-GBM cell line (Happold et al. 2012). To check the cytotoxic effect of rabeprazole and temozolamide on LN18 cells we performed MTT and clonogenic assay. The MTT results showed dose dependent cell growth inhibition upon rabeprazole treatment. The half-inhibitory concentration (IC_{50}) of rabeprazole was observed at ~120 μM **(Figure 7A)**, but we did not find any significant cell growth inhibition at 200 μM of TMZ concentration **(Figure 7B)**. Combined exposure of rabeprazole and TMZ on LN18 cells revealed significant cell growth inhibition at 50 μM rabe+100 μM TMZ dose **(Figure 7C)**. Results obtained from clonogenic assay showed 2.8% and 27% colony reduction at 50 μM and 100 μM of rabeprazole dose respectively, in comparison to the vehicle **(Figure 7D, E)**. We also observed 12.17% and 15.66% colony reduction at 50 μM and 100 μM of TMZ concentration respectively, in comparison to the vehicle control, while the combined treatment of rabeprazole and TMZ at 50 μM rabe+100 μM TMZ and 100 μM rabe+50 μM TMZ showed 46% and 67% colony reduction respectively **(Figure 7D, E)**. The significant reduction in colony count was observed in combined treatment confirming TMZ sensitization. In addition, we also showed significant inhibition of vimentin and β-catenin expression levels by western blot in a combined dose of rabeprazole and TMZ at 50 μM rabe+100 μM TMZ concentration as compared to the vehicle and 50 μM of rabeprazole or 100 μM of TMZ in C6 and LN18 cell lines **(Figure 7F-I)**.

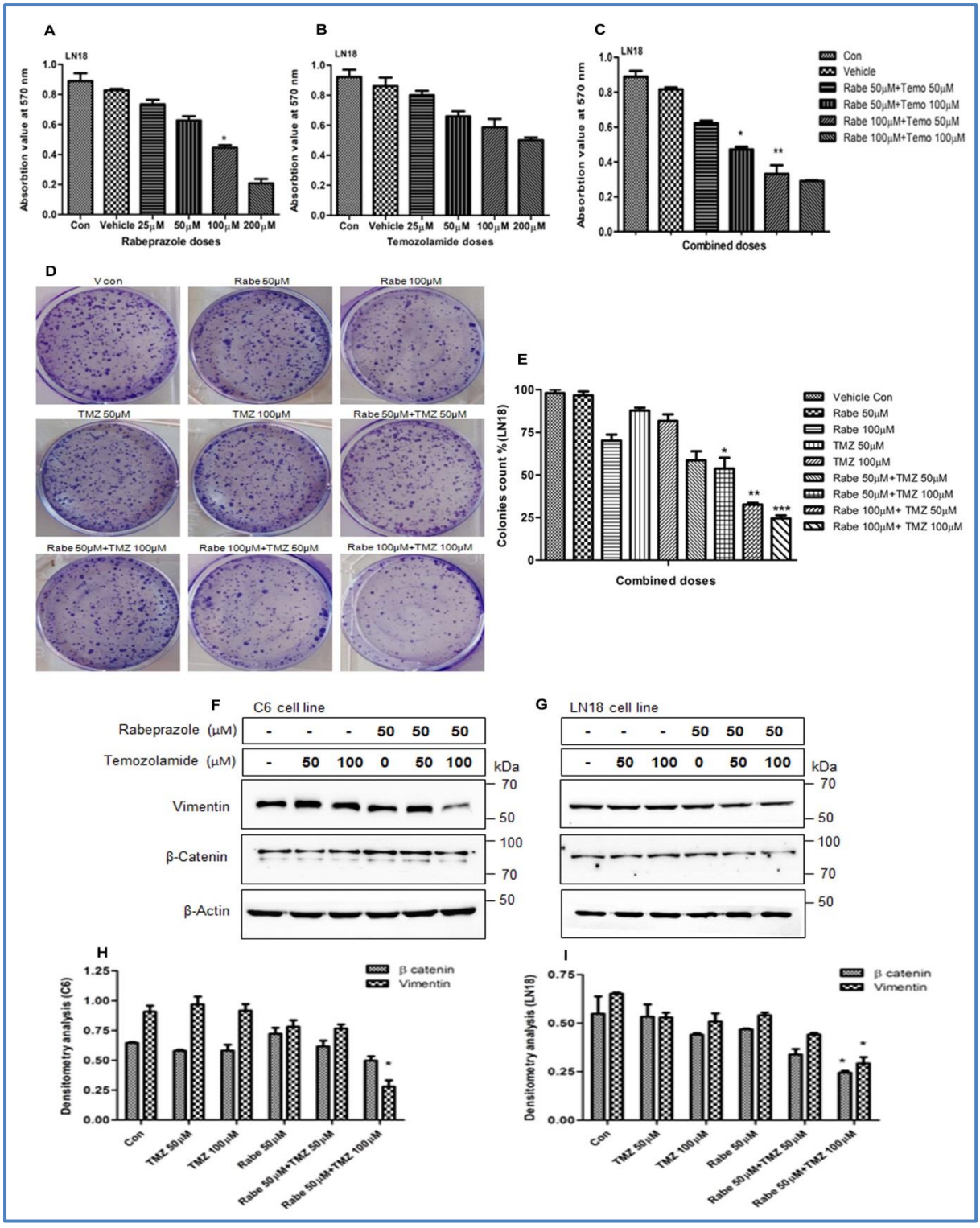

Figure 7. TMZ sensitization by rabeprazole on a TMZ resistant cell line. (A, B) MTT results using LN18 cell line show dose dependent cell growth inhibition upon rabeprazole treatment, we did not find significant growth inhibition even at 200 μM of TMZ concentration. **(C)** Combined treatment of rabeprazole and TMZ reveals significant cell growth inhibition at 50μM rabe+100 μM TMZ dose. **(D, E)** The clonogenic images show dose dependent colony reduction after rabeprazole treatment. TMZ treatment at 50 μM and 100 μM of concentration showed only 12.17% and 15.66% colony reduction respectively.

The combined treatment of rabeprazole and TMZ at two different doses indicated in the figure shows 46% and 67% colony reduction. **(F, G)** Representative western blots and **(H, I)** its densitometry quantification show significant inhibition of vimentin and β-catenin expression level in a combined dose of rabeprazole and TMZ at 50 μM rabe+100 μM TMZ concentration compared to the vehicle and 50 μM of rabeprazole or 100 μM of TMZ dose in C6 and LN18 cell lines. Bars indicate SEM. (*) indicate $p \leq 0.05$, (**) = $p \leq 0.02$, (***) = $p \leq 0.001$.

2.4 Discussion

Cancer malignancy and drug resistance mostly contributes to the cancer-associated deaths. EMT is a well-studied developmental regulatory pathway critical for embryonic development and wound healing process, further, its presence in cancer aggravates cancer invasion, migration and drug resistance (Hanahan and Weinberg 2011; Polyak and Weinberg 2009). Although, EMT is well documented in other cancers, its recognition in glioma is relatively new with a different terminology known as EMT like process or glial to mesenchymal transition (Iser et al. 2017; Mahabir et al. 2014). In the present study, we have investigated the clinical importance of EMT like process in human glioma and validated its therapeutic potential by rabeprazole with an intention of repurposing rabeprazole as an anticancer drug. The expression analysis of EMT associated proteins in glioma tissues revealed negative expression of E-cadherin, differential weak expression of N-cadherin and upregulation of GFAP, vimentin and β-catenin in different grades of glioma as compared to the control tissues. The negativity of E-cadherin and increased expression of GFAP, vimentin and β-catenin is associated with glioma malignancy and dismal prognostic outcomes. Interestingly, the correlation study indicated a positive correlation between GFAP, vimentin and β-catenin and a negative correlation between N-cadherin and E-cadherin with vimentin. These observations support EMT 'all or nothing' event and justify the independency of cadherin switch or noncanonical EMT process in glioma in the notion of previous studies (Iser et al. 2017; Utsuki et al. 2002).

Multiple evidences support the concept that proton pumps aid in epithelial to mesenchymal transition by modulating the pH of tumor microenvironment in addition to the molecular aberration and hypoxia (Lee and Tannock 1998; Marcucci et al. 2014; Martinez-Zaguilan et al. 1996; Robertson et al. 2004; Sahlgren et al. 2008). So far, various inhibitors have been used to target EMT but due to their high toxicity they could not be used as therapeutic agents (Marcucci et al. 2016). The first observation for the anticancer activities of PPI was reported using gastric cancer model (Yeo et al. 2004). Since, then several studies (Azzarito et al. 2015; Canitano et al. 2016; Feng et al. 2016; Song et al. 2016; Zhang et al. 2014) have demonstrated antineoplastic and chemosensitizing ability of PPI. Notably, P. Breedveld et al 2014 (Breedveld et al. 2006) and S. Ferrari et al 2013 (Ferrari et al. 2013) showed that pantoprazole inhibits BCRP and chemosensitization activity, contributing to an improved CNS delivery of imatinib, thereby suggesting that PPI may increase the efficacy of chemotherapy through a mechanism other than pH modulation. Recently, anti-EMT efficacy and chemosensitization ability of PPI was reported in gastric cancer (Feng et al. 2016; Zhang et al. 2015) that persuaded us to investigate the anticancer and anti EMT potentials of rabeprazole on GBM cell lines and C6 rat glioma model.

Our data revealed that the rabeprazole exposure induced cell death and arrested cell cycle at G0/G1 phase. Rabeprazole treatment increased caspase-3 and cleaved PARP levels together with the altered anti and proapoptotic proteins (Bcl-2, Bax and p53) that confer apoptosis. Previous studies showed that the PPI treatment induces cell death via pH dependent caspase activation or by ROS triggered mitochondrial apoptosis (Canitano et al. 2016; De Milito et al. 2010; De Milito et al. 2007; Simon et al. 2000). In agreement with these reports, we also observed that the rabeprazole treatment induced ROS levels and depolarized mitochondrial integrity as indicated by RH-123 staining, and cytochrome-c release in cytosolic fractions.

EMT in cancer cells enhanced cellular plasticity and resistance to apoptosis and drug treatment (Thiery 2002). We showed rabeprazole administration inhibited cell colonization and cell migration ability together with EMT suppression by targeting Wnt/β-catenin and NF-kB signaling. In the earlier report, we showed Wnt/β-catenin signaling activation in astrocytoma (Sareddy et al. 2009). The involvement of Wnt/β-catenin signaling in EMT induction has been studied in other cancers (Kim et al. 2002; Zhang et al. 2013). Here, we have demonstrated that the rabeprazole exposure significantly inhibited Akt and Gsk-3β phosphorylation, which in turns suppresses EMT transcriptional inducer snail and β-catenin which is in agreement with earlier studies (Bachelder et al. 2005; Zhang et al. 2015). The *in-vivo* data revealed restricted tumor growth with augmented animal survival in the confluence of EMT inhibition corroborating clinical observations disclosing that the increased EMT proteins exhibit patient's poor survival.

Further, we showed rabeprazole treatment targets NF-kB signaling and affirmed with TNF-α induced EMT inhibition by attenuating NF-kB-P65 expression and nuclear translocation, in consistence with previous reports which shows TNF-α induced EMT pathway through NF-kB activation (Li et al. 2012; Wang et al. 2013). Collectively, these evidences demonstrated the anti EMT efficacy of rabeprazole by targeting Wnt/β-catenin or NF-kB signaling; In tandem we also presented an efficient inhibition of STAT3, a master regulator of the EMT pathway *in-vitro* and *in-vivo* thereby, displaying rabeprazole credential as an anticancer and anti EMT drug.

Emerging studies report TMZ resistance in malignant glioma impedes GBM therapeutic interventions (Happold et al. 2012; Ramirez et al. 2013). In fact, the recent evidences identified that PPI pre or combined treatment can sensitize chemoresistance (Azzarito et al. 2015; Song et al. 2016). Coinciding with anti EMT and anticancer efficacy of rabeprazole, we also demonstrated the TMZ sensitization by rabeprazole on TMZ resistant cell line, as evident from cell cytotoxic and clonogenic assays.

Simultaneously, we also showed the significant inhibition of EMT protein vimentin and β-catenin in combined dose of rabeprazole and TMZ.

2.5 Conclusion

In conclusion, this study demonstrated the increased EMT proteins in glioma grades and their association with the advancement of the disease and clinical outcomes. Further, *in-vitro* and *in-vivo* data confirmed that the rabeprazole treatment restricted glioma growth, malignancy and TMZ resistance through EMT suppression. Together, we provide the proof of concept that rabeprazole could be a safe and effective candidate for therapeutic intervention of malignant glioma.

Additional Information

Study Approval

Institutional Ethics Committee (IEC) (reference number UH/IEC/2016/180) and Institutional Animal Ethics Committee (IAEC) (reference number UH/IAEC/PPB/2017-I/P7), University of Hyderabad, Hyderabad (500 046) approved all procedures involving human tissue and animal related experiments.

Supporting Information

Supplementary Tables

Table S1. EMT associated proteins in human astrocytoma

Glioma grades	GII (n=16)	GIII (n=12)	GIV (n=16)	Total (n=44)
E-cadherin (- ve)	13 (81.25%)	11 (91.66%)	14 (87.5%)	38 (86.36%)
GFAP (+ ve)	14 (87.5%)	11 (91.66%)	14 (87.5%)	39 (88.36%)
Vimentin (+ ve)	10 (62.5%)	07 (58.33%)	12 (75%)	29 (65.90%)
β-catenin (+ ve)	12 (75%)	07 (58.33%)	13 (81.25%)	32 (72.72%)

EMT like process accessed at protein levels using EMT biomarkers; (+ve/-ve) depicts protein expressions.

Table S2. Clinical correlation between EMT proteins in human astrocytoma			
	Vimentin expression profile		Fischer's exact test
	(+)	(-)	
E-cadherin (+)	09.09% (04)	04.54% (02)	p=0.67
E-cadherin (-)	56.81% (25)	29.54% (13)	
	Vimentin expression profile		
	(+)	(-)	
GFAP (+)	56.81% (25)	31.81% (14)	p=0.43
GFAP (-)	09.09% (04)	02.27% (01)	
	Vimentin expression profile		
	(+)	(-)	
β-catenin (+)	59.09% (27)	11.36% (05)	p=0.0005
β-catenin (-)	06.81% (02)	22.72% (10)	
The association between Vimentin with E-cadherin, GFAP and β-catenin were analyzed in astrocytoma tissue (n=44) by Fischer's exact test; (+)/ (-) represents positive or negative expression of a protein.			

Supplementary Figures

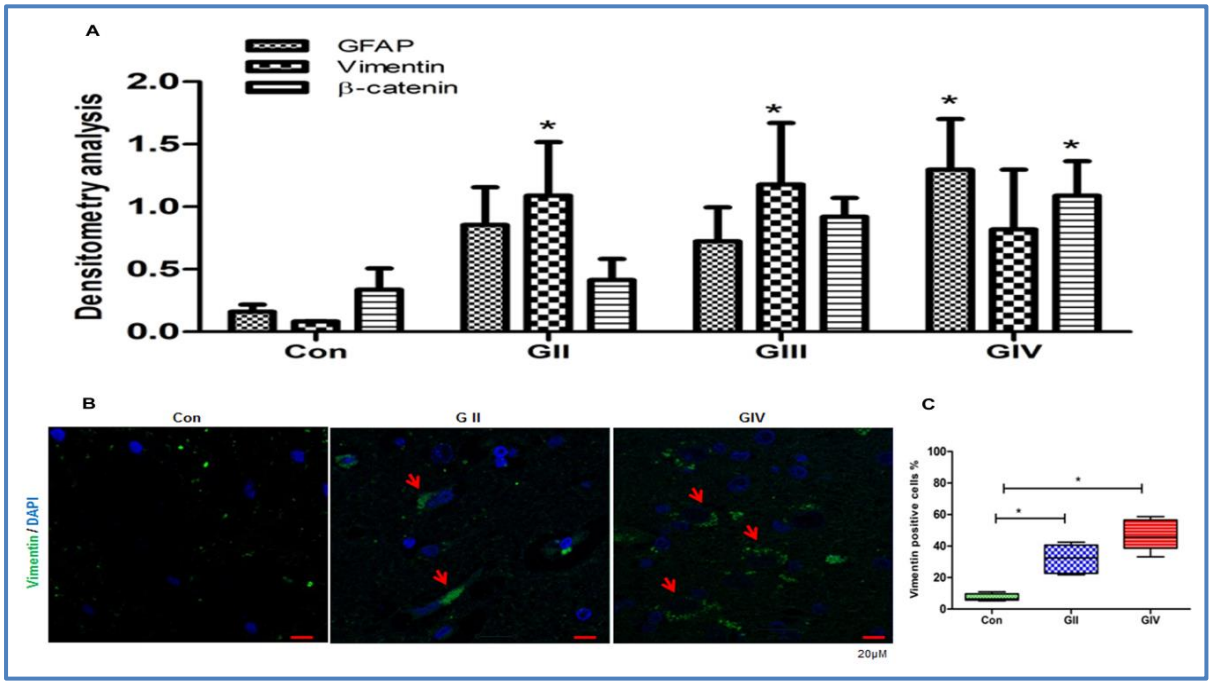

Figure S1. Increase EMT proteins in glioma grades. (A) The densitometry graph indicates the significant increase of EMT protein in glioma grades (n=44) compared to the control tissues (n=7). **(B)** IF image show significant increase (p=0.001) of cytosolic vimentin in glioma grades as compared to the control tissues.

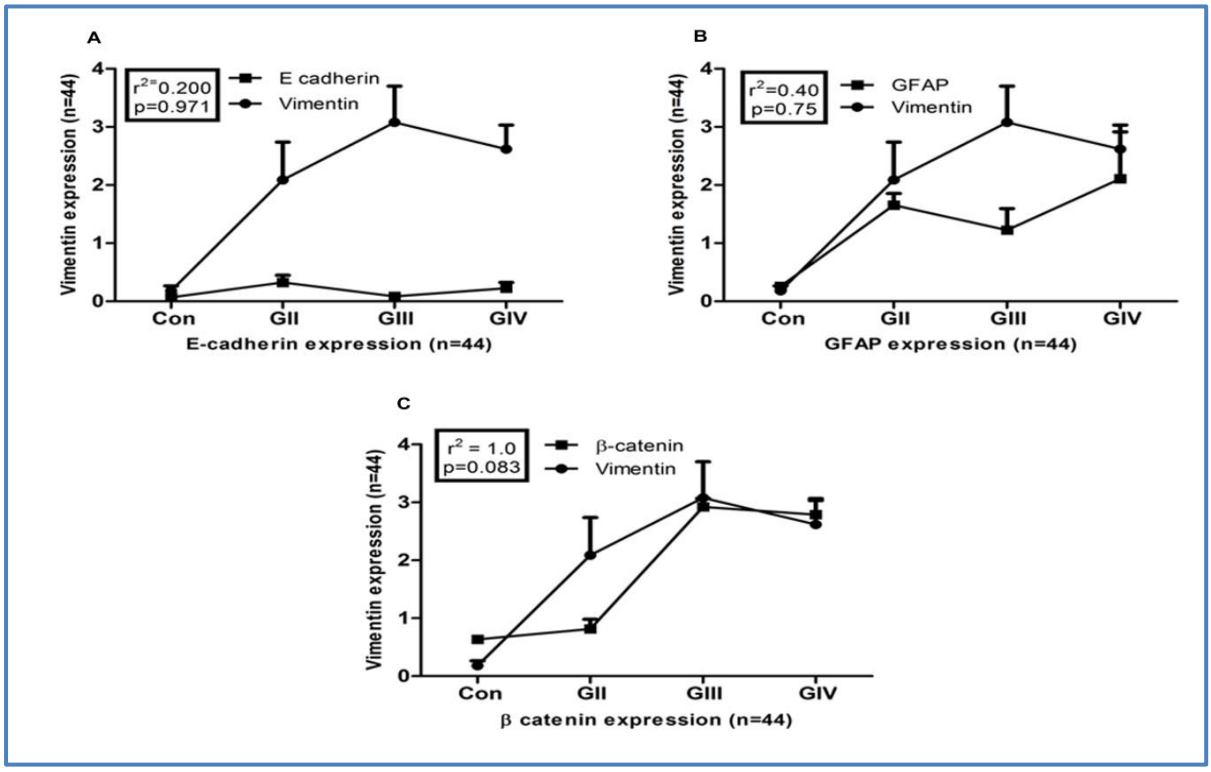

Figure S2. Correlation study of EMT proteins in glioma grades. Graph depicts the correlation of E cadherin **(A)**, GFAP **(B)** and β-catenin **(C)** with vimentin in different grades of glioma by spearman correlation coefficient (r^2). The C.C. values range from -1 to +1. The C.C value of "-1" shows strong negative correlation. C.C value "+1" shows strong positive correlation and C.C value "0" indicates no correlation.

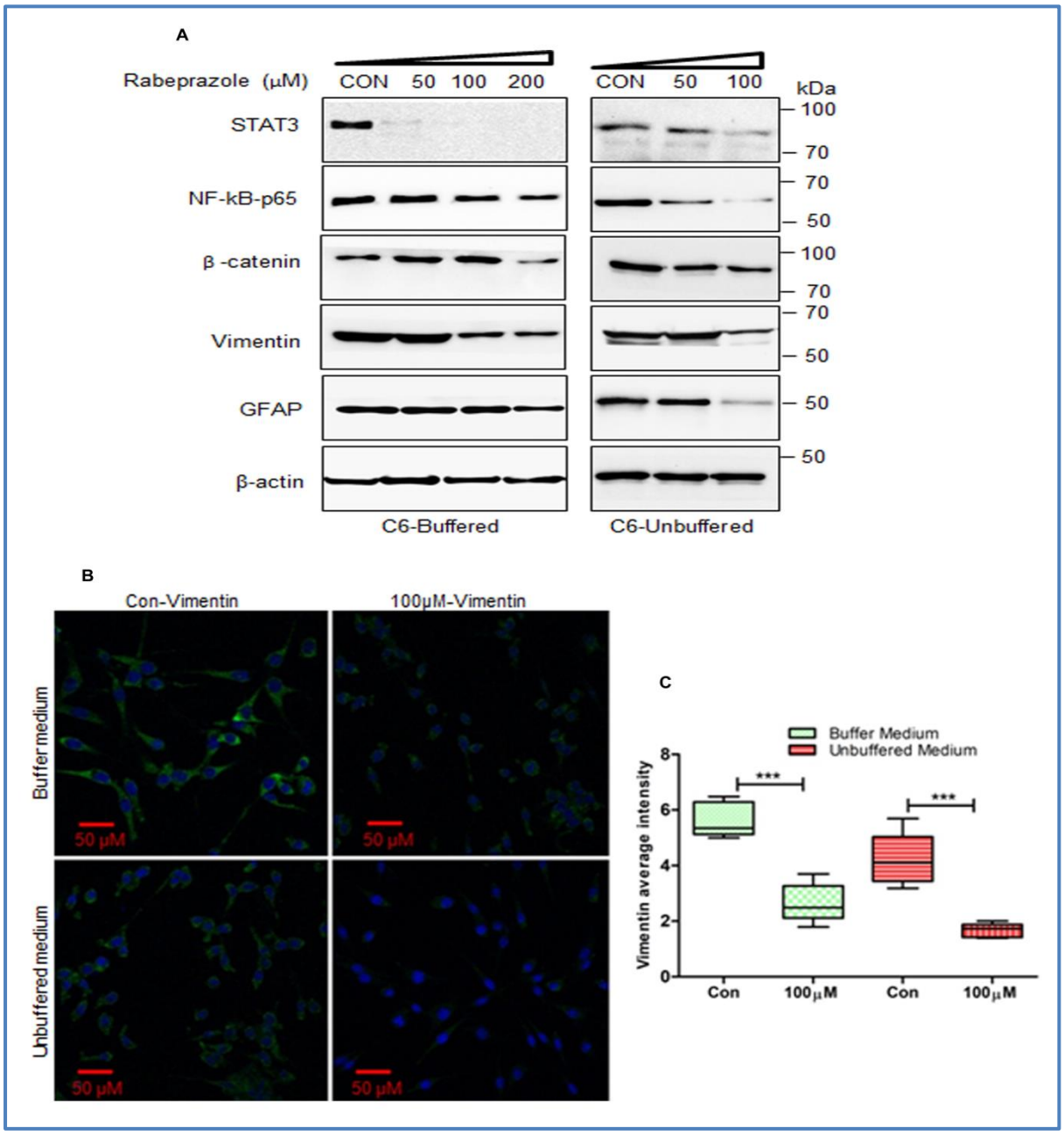

Figure S3. Rabeprazole suppress EMT proteins and its transcriptional regulators. Western blots showed GFAP and EMT proteins vimentin and β-catenin inhibition together with EMT regulator NF-*κ*B-P65 and STAT3. **(H)** The IF images evidence vimentin suppression upon rabeprazole treatment. **(I)** Graph reveals significant vimentin inhibition at 100 μM of rabeprazole concentration in both conditions.

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Chapter 3

Molecular dissection of breast cancer metastasis suppressor 1 (BRMS1) in astrocytoma and its etiological connection with EMT

Objective 1

- **Distinct expression and role of BRMS1 in *mut*P53 GBM**

Objective 2

- **Clinical and therapeutic relevance of BRMS1 to EMT in *mut*P53 GBM**

Abstract

Glioblastoma multiforme (GBM) is a noted malignancy of the central nervous system (CNS) tumor with a poor prognosis among all malignant forms. Breast cancer metastasis suppressor 1 (BRMS1) suppresses metastasis in multiple tumors, but its clinical and molecular importance in *mut*P53 GBM is not known. Here, we have investigated the clinical and therapeutic significance of BRMS1 in *mut*P53 GBM and its etiological relevancies with EMT. BRMS1 expression was evaluated in human astrocytoma tissue (n=74) and analyzed with various clinicopathological parameters, its downstream targets and EMT proteins. The *BRMS1* siRNA transfection study was performed to elucidate the role of BRMS1 and its concomitant signaling in GBM malignancy and chemoresistance. Our findings revealed overexpression of BRMS1 in malignant astrocytoma associated with pathological grading and patient's poor survivability. We observed the positive correlation between BRMS1 and *mut*P53 and/or EMT proteins in GBM tissues which may extort GBM malignancy and poor prognosis. The *in-vitro* BRMS1 siRNA study revealed reduced cell growth, cell migration and increased apoptosis in *mut*P53 GBM cell line. Also, The *BRMS1* knockdown suppressed EGFR-AKT and EMT signaling together with NF-$\varkappa$B expression in *mut*P53 cells. The transcript profiling disclosed a positive and negative correlation of *uPA* and *ING4* with *BRMS1* respectively. Altogether, we report an upregulation of BRMS1 in *mut*P53 glioblastoma worsens GBM malignancy and patient's survivability in confluence of EMT induction. The *BRMS1* knockdown restricted cell growth, cell migration and sensitized apoptosis and TMZ resistance by modulating EGFR-AKT and EMT signaling in *mut*P53 GBM cells, suggesting BRMS1 can be a prognostic and therapeutic target for *mut*P53 GBM.

3.1 Introduction

Astrocytoma is one of the most frequently reported astrocytes derived primary central nervous system (CNS) tumors that attributes 75% of primary CNS tumors with 60% malignancy (Ostrom et al. 2016). According to the WHO-2007 histopathological classification, astrocytomas are divided into four grades, Grade I to Grade IV (Louis et al. 2007). Grade I (pilocytic astrocytomas) and Grade II (diffuse astrocytoma) are low grade astrocytoma with 5-6 years median survival and are usually cured by surgery (Ostrom et al. 2016; Wen and Kesari 2008). Compared to that, the treatment outcome for high grade astrocytoma such as Grade III (anaplastic astrocytoma) and Grade IV (glioblastoma multiforme) is very low, with a median survival of 18 months post therapy (Furnari et al. 2007; Wen and Kesari 2008). Recently, molecular classification included IDH mutation to facilitate the clinical outcomes (Louis et al. 2016). In spite of the current diagnostic and therapeutic advances in GBM treatment, the five-year post diagnosis survival remains same, ≤5.5% (Ostrom et al. 2016).

Breast cancer metastasis suppressor gene 1 (*BRMS1*) is a noted metastasis suppressor gene (MSG) that can cease metastasis without affecting tumor growth (Welch et al. 2000). *BRMS1* was initially identified through differential display analysis (Samant et al. 2000). The transfection study of *BRMS1* cDNA into breast cancer cell lines demonstrated metastasis suppression without affecting tumourigenicity (Samant et al. 2000). Although it has been more than a decade from the discovery of *BRMS1* to its recognition as a potential tumor regulator gene, its expression and clinical significance are discrepant (Bucciarelli et al. 2018; Kodura and Souchelnytskyi 2015; Yang et al. 2016; Zhang et al. 2006; Zhang et al. 2014). Notably, very limited information in astrocytoma (Mei et al. 2014) advocates the necessity of further investigations of BRMS1 in astrocytoma.

P53 is a well-studied tumor suppressor gene known for genome stability (Levine et al. 1991). In astrocytoma P53 missense mutation is frequent and its expression is linked with GBM malignancy (Levine et al. 1991; Watanabe et al. 1997). In fact, the P53 expression increases with tumor advancement from benign (GII: 18-46%) to malignant (GIII: 29-57% and GIV: 49-70%) (Sarkar et al. 2002; Watanabe et al. 1997). Moreover, P53 is a potential driver gene that can modulate the expression or function of other passenger genes in various cancers, including astrocytoma (Levine et al. 1991; Muller and Vousden 2013; Watanabe et al. 1997). Recently, Hall et al. 2014; and Liu et al. 2015 evidenced the alteration of BRMS1 in *mut*P53 or loss of P53 gene in epithelial cells may promote mesenchymal phenotype leading to the cell migration and cell invasion (Hall et al. 2014; Liu et al. 2015).

Considering these facts we demonstrated the BRMS1 expression in astrocytoma grades and its association with various pathological features and patient's survivability. We also showed the connection between BRMS1, *mut*P53 and EMT proteins in astrocytoma tissues. Further, the *BRMS1* knockdown study evidences its possible role in GBM cell growth, cell migration and temozolomide (TMZ) resistance. The *BRMS1* silencing suppresses EGFR-AKT and EMT signaling in *mut*P53 cell line. Furthermore, to provide the molecular insight of BRMS1 we performed transcript profiling of BRMS1 with its downstream targets urokinase plasminogen activator (UPA) and inhibitor of growth family-4 (ING4)(Cicek et al. 2009; Li and Li 2010); as both genes are also associated with astrocytoma malignancy(Kit et al. 2017; Klironomos et al. 2010).

3.2 Materials and Methods

3.2.1 Tumor specimens: collection and processing.

In the present study total 74 astrocytoma tissues of different grades and 12 non tumorous (temporal epileptic tissues) brain tissues were collected from the Krishna institute of medical science (KIMS), Secunderabad, India. In total biopsies, 22 tissues were Grade II (GII), 22 tissues were Grade III (GIII) and 30 tissues were Grade IV (GIV). The grading was based on WHO-2007 histopathological classification (Louis et al. 2007). After surgical resection, these tissues were immediately frozen in liquid nitrogen and then stored at -80°C till further analysis. Written informed consent was obtained from all participating individuals and study was approved by Institutional ethics committee (IEC), University of Hyderabad, Hyderabad (TS).

3.2.2 Cell culture

GBM Cell lines LN18, U373 and U87 were procured from NCCS-Cell repository Pune, India. T98 cell line was a kind gift from Dr. Ellora Sen (National Brain Research Center, Gurgaon, India). U373, LN18, and T98 cell lines are *mut*P53 while U87 is a *wt*P53 cell line (Van Meir et al. 1994). All cells were authenticated and free from mycoplasma or bacterial contaminations. Cells were grown in Dulbecco's modified Eagle's medium (DMEM) (AL007A), supplemented with anti-anti (15240062) and 10% fetal bovine serum (RM9955). All cell lines were maintained at 37^0C in 5% CO_2 incubator.

3.2.3 RNA isolation and Reversed transcribed-qPCR (RT-qPCR)

Total RNA was isolated from clinical tissues or cell lines using trizol (T9424-sigma) reagent as per the manual instruction. Quantified with NanoDrop, 1-5 µg of RNA was used for **cDNA synthesis** using **BluePrint 1st Strand cDNA Synthesis Kit** (#6115T). Reaction mixture for **cDNA** synthesis was incubated at 30°C for 10 min and 42°C for 60 min, and the enzyme was inactivated by heating at 95°C for 5 min. Quantitative-PCR (qPCR) was performed by using

the SYBR® Premix Ex Taq™ (RR420A) with Applied Biosystems 7500 Fast Real-Time PCR System. Diluted cDNA was used in a 20 µl reaction in triplicate for each gene. Primers sequences are enlisted in supplementary table 1 (Table S1).

3.2.4 Western blotting

Protein was extracted from the tissues biopsies and cell lines by earlier described method (Sareddy et al. 2012). Proteins were separated by SDS-PAGE and transferred onto nitrocellulose membrane. The membranes were blocked with 5% nonfat milk solution or 5% bovine serum albumin at room temperature for 1 hour. After blocking membrane was probed with specific primary antibodies as BRMS1 (ab134968) or β-actin (ab8227) at 4°C overnight, followed by incubation with horseradish peroxidase (HRP) conjugated secondary antibody (#7074). Immunoreactivity was visualized by Bio-Rad chemidoc imaging system.

3.2.5 Immunohistochemistry (IHC) and Immunofluorescences (IF)

Tissue sections of 5-10µm were prepared by cryotome (LEICA-1850) and stored at room temperature. Before staining tissue section were dewaxed by heating slides at 100°C for 15min and 3 washes in xylene 5 min each further rehydrated by simultaneous 5 min washes in 100, 95, and 75 and 50% ethanol and distilled water. Antigen retrieval was done by 18 min cooking in Tris/EDTA (pH 9.0) buffer and Immunohistochemical staining as per the DAKO LSAB+ KIT manual. The tissue sections were thoroughly washed with phosphate buffered solution (PBS) in each step, visualized with 3,3'-diaminobenzidine tetrahydrochloride, and lastly counterstained with hematoxylin as described in previous report (Sareddy et al. 2009). For IF Briefly, tissues sections were incubated with protein-specific primary and FITC-conjugated secondary antibody for one hour at RT. After antibody incubations, sections were washed with PBS and counterstained with a vectashield mounting medium. Images were analyzed through the confocal

microscope. The IHC quantification of positively stained/unstained cells were performed by previously described method (Lee et al. 2017). The expression was categorized into negative, low, moderate and high in following manner (0-5% stained cells- negative, >5-20% stained cells- low, >20-50% stained cells- moderate and >50% stained cells- high).

3.2.6 Si-RNA transfection study

For siRNA mediated knockdown study, siRNA specific for *BRMS1* (Sense seq.: 5'GAU-GGA-UGA-UGA-GGA-CUA-U3, Anti sense seq.:5'AUA-GUC-CUC-AUC-AUC-CAU-C 3') was design and synthesized from Eurogentec Belgium (SR-NP001-004-siRNA Duplex SePOP). Commercially available non silencing siRNA was purchased from Qiagen (#1022076). Polyplus-jetPRIME transfection reagent (Illkirch cedex- France) was used for target and non target siRNA treatment in accordance with manual instruction. Maximum transfection efficiency was calculated at 15 nM of siRNA concentration. The *BRMS1* silencing was confirmed at mRNA and protein levels upon 48 hours and 72 hours of siRNA treatment respectively.

3.2.7 MTT and Clonogenic assays

Approximately 5 × 10^3 cells/ well were seeded in 96 wells plate and kept in a CO_2 incubator for overnight growth. When cells reached to 50-60% confluency, *BRMS1* siRNA and non targeting siRNA treatment were given as per the jet PRIME kit manual. After 24 hours or 48 hours of growth 20 µl of MTT (5mg/ml) was added to each well of 96 wells plate and 4 hours incubation in CO_2 incubator. After the incubation, 50 µl of DMSO was added to each wells and absorbance was taken at 570 nm by a multiple plate reader (Tecan Infinite 200). For the colony formation assay, approximately 500-1000 cells in single cell suspension were seeded in each well of six well plates. After 24 hours of growth the *BRMS1* siRNA and non targeting siRNA treatment were given. Upon 24 hours of siRNA exposure medium was changed and kept in CO_2

incubator. Cells were grown until the colonies were visible. Colonies were fixed with 4% paraformaldehyde (PFA) for 10 minutes at room temperature and stained with 1% crystal violet. Images were taken with Olympus digital camera (Olympus, Japan). Data were analyzed through previously described procedure (Du et al. 2004).

3.2.8 Transwell invasion assays

Cell invasion was checked by transwell inserts (polycarbonate membrane of 8-micron pore size) coated with 2% gelatin and collagenase. Upon 24 h of BRMS1 siRNA treatment, the cells were seeded in transwell inserts in serum free media and kept in a CO_2 chamber for 12 h incubation. FBS was used as a chemo attractant. After 12 h of growth, inserts were carefully removed and washed with PBS twice. Traversed cells were fixed in methanol and stained with 1% crystal violet. To analyse results, six individual fields of traversed cells of the membrane were photographed and counted.

3.2.9 Statistical Analysis

Statistical analyses were performed using prism graph pad version 5.01. The differences between the data sets were calculated by one-way ANOVA and the differences between two variables were determined by student-t test. The survival statistics were analyzed by Kaplan-Meier method and the clinicopathological associations with *BRMS1* expression were evaluated by Freeman–Halton, extension of the Fischer exact test. Correlation between *BRMS1* and its downstream gene expression in different grades of astrocytoma were analyzed by Pearson correlation coefficient. The Correlation Coefficient value (r^2) = +1 was considered as strong positive and r^2 = -1 indicates negative correlation while r^2= 0 represents no correlation. The continuous data were represented as mean ± SEM. The *p value ≤ 0.05 were considered as statistically significant.

3.3 Results

3.3.1 BRMS1 overexpression in malignant astrocytoma tissues

To discern the clinical significance of BRMS1 in astrocytoma we first evaluated the mRNA expression in different grades of astrocytoma (n=74) and control brain tissues (n=12). Results showed significantly high mRNA expression in grade IV compared to grade II (*p≤0.002) and control brain tissues (*p≤0.004) **(Figure 1A)**. The mRNA expression increased with grade progression, but the upregulation between GII and GIII or either when compared with the control was not significant. The western blot demonstrates that BRMS1 protein was upregulated in malignant astrocytoma as compared to the control tissues and lower grades **(Figure 1B)**. Densitometry analysis showed significant increase of BRMS1 protein in GIV compared to the control tissues (*P≤0.0005), GII (*P≤0.0002) and GIII (*P≤0.0001) **(Figure 1C)**. β-actin was used for equal loading. The immunohistochemistry (IHC) staining was consistent with qPCR and western blot data showing high BRMS1 positivity in GIV (>60% stained cells) as compared to the control (<20% stained cells), GII (<35% stained cells) and GIII (<40% stained cells) **(Figure 2A, B)**.

3.3.2 Increased BRMS1 expression associated with poor prognosis in GBM

To determine the clinical significance, the BRMS1 expression pattern is divided into three groups (negative, low and high) based on the degree of expression evident from western blots and IHC. Out of 74 cases, 66 cases were analyzed for Kaplan-Meier survival statistics; follow-up details were not available for the remaining 8 cases. Kaplan-Meier survival curve showed patients with high BRMS1 expression revealing poor prognosis in comparison to the negative and low BRMS1 expressing patients **(Figure 2C)**. The log-rank (Mantle-Cox) test showed significant survival distribution (*p≤0.0001) between the mentioned groups and the median survival of BRMS1 overexpressed astrocytoma patients is 16 months.

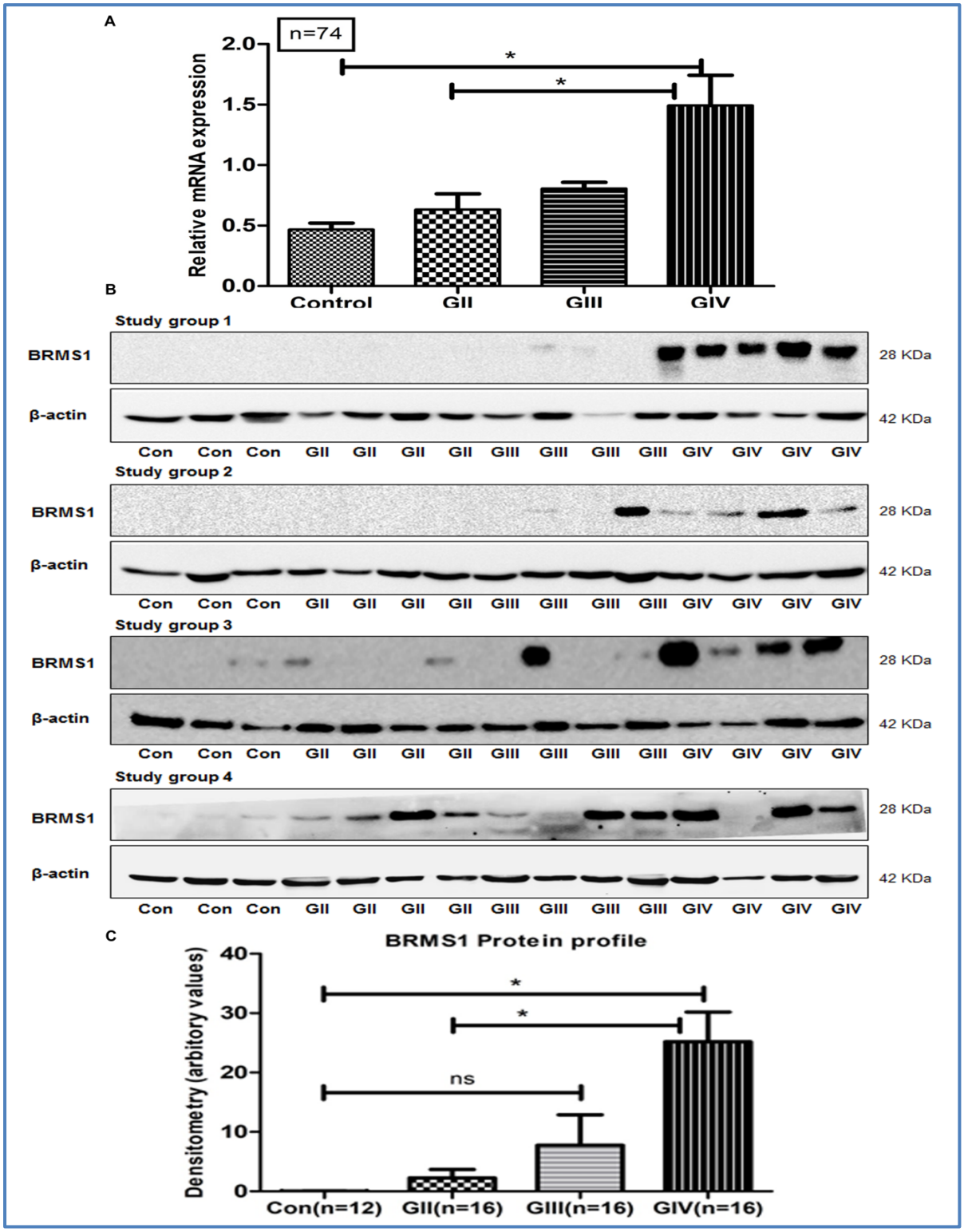

Figure 1. BRMS1 overexpression in malignant astrocytoma. (A) The mRNA expression was analyzed by qPCR indicating the increased mRNA level in astrocytoma grades compared to the control; GIV tissue shows significant upregulation in comparison to the control. **(B)** Western blots show high BRMS1 protein expression in high grade astrocytoma (GIII and GIV) compared to the low grade and control. **(C)** Densitometry analysis reveals significant expression in GIV and GIII compared to GII and control. The bar represents SEM and p-value ≤ 0.05 was considered as statistically significant.

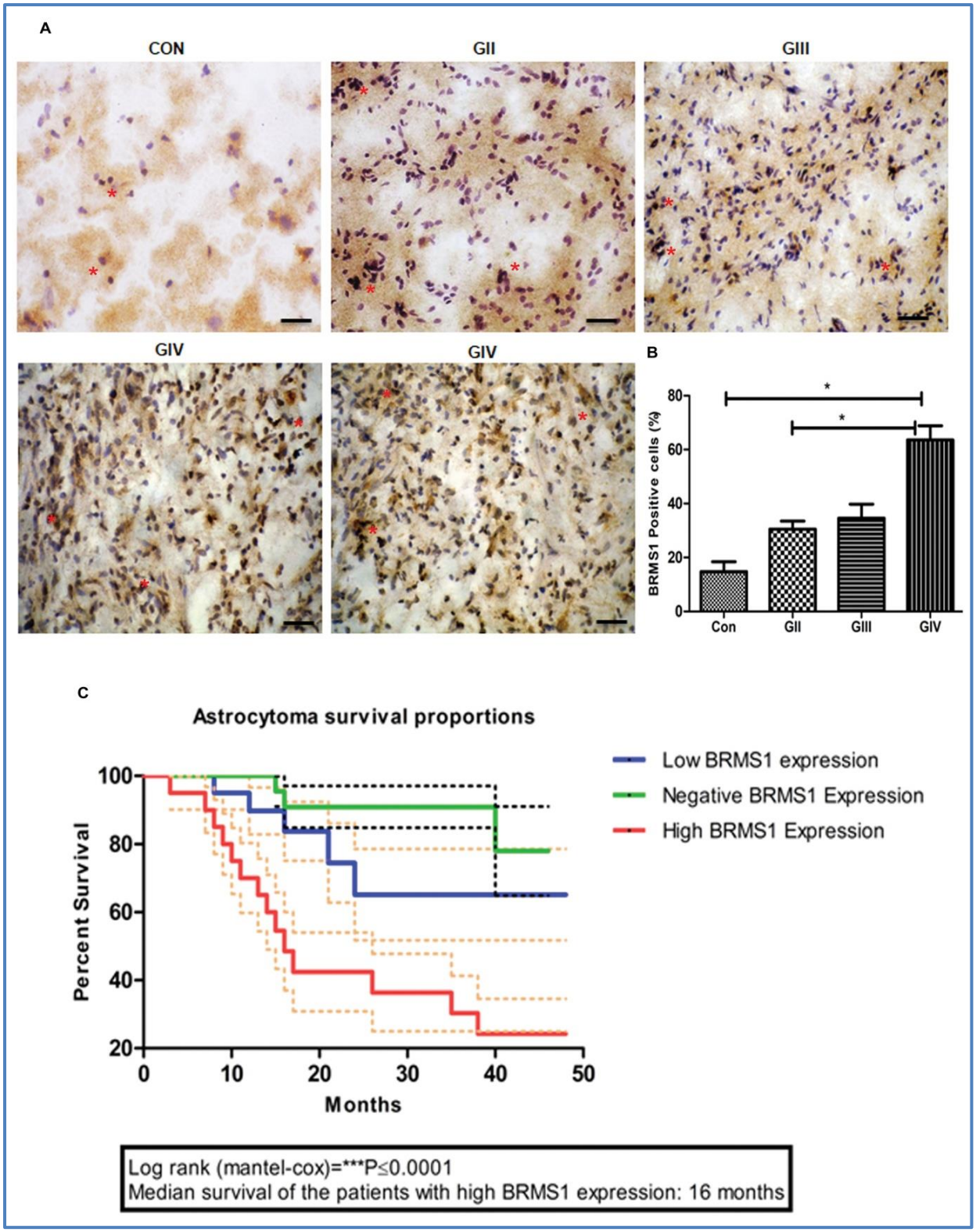

Figure 2. Increased BRMS1 expression associated with patient's poor survival. (A, B) Immunohistochemistry images depict high BRMS1 positivity (>60% staining) in GIV tissue compared to the GIII (<40% staining), GII (<35% staining), and control (<20% staining) tissue. **(C)** The Kaplan-Meier survival graph shows high BRMS1 expression inflicts poor prognosis; Log-rank test reveal significant survival statistics ($p \leq 0.0001$).

3.3.3 BRMS1 expression is positively correlated with astrocytoma grades

The BRMS1 expression is correlated with the various clinicopathological parameters including age, gender and different grades of astrocytoma as summarized in **Table 1**. In total 74 clinical biopsies, we found BRMS1 expression in 45 (60.81%) tissues. Out of 45 BRMS1 expressing tissues, 26 tissues (57.77%) were identified with high BRMS1 expression and 19 tissues (42.22%) with low BRMS1 expression. Twenty –nine (39.18%) tissues were BRMS1 negative. The BRMS1 overexpression was significantly correlated with astrocytoma grade progression (*p≤0.004). However, we did not find significant correlation between BRMS1 expressions with patients' age (p≤0.71) or gender (p≤0.30) **Table 1**.

Table 1: The correlation between BRMS1 expression with various clinicopathological features

Features	**BRMS1 Expression Profile (N=74)**			**chi-square test**
	Low (n=19)	**High (n=26)**	**Negative (n=29)**	
Gender				
Male (n=45)	12 (17.77%)	17 (33.33%)	16 (48.88%)	ns
Female (n=29)	07 (20.68%)	09 (48.27%)	13 (31.03%)	
Age (years)				
≤45 (n=53)	11 (35.18%)	20 (25.92%)	22 (38.88%)	ns
>45 (n=21)	08 (23.80%)	06 (33.33%)	07 (38.09%)	
Histopathological Grades (WHO-2007, Classification System)				
GII (n=22)	05	03	14	p=0.004
GIII (n=22)	09	06	07	
GIV (n=30)	05	17	08	

3.3.4 Concomitant expression of BRMS1 and *mut*P53 worsens astrocytoma malignancy and survival

The frequent P53 mutation has been linked to astrocytoma malignancy (Levine et al. 1991; Watanabe et al. 1997). To identify the relationship between P53 and BRMS1 in

astrocytoma, we checked P53 transcript and protein in BRMS1 expressing astrocytoma tissues. The qPCR **(Figure 3A)** and western blot **(Figure 3B)** data showed high P53 transcript and protein levels in BRMS1 expressing astrocytoma tissues, particularly P53 expression was quite similar to the BRMS1 expression in GIV tissues **(Figure 3A, B)**.

The correlation study illustrated the positive correlation between BRMS1 and P53 at transcript r^2= 0.9701; *$P<0.0299$ **(Figure 3C)** and protein levels r^2= 0.9508; *$P<0.049$ **(Figure 3D)**. Since BRMS1 nuclear localization is associated with invasive behavior of the cancer (Riker and Samant 2012), we also checked the BRMS1 and P53 localization in tissue section of low and high grade of astrocytoma and control brain tissue section by IHC and IF (immunofluorescence). The IHC staining showed high nuclear positivity of BRMS1 (>70% staining) and P53 (>60% staining) in GIV as compared to the GII (<40% staining) control tissues (<10% staining) (**Figure 4A, B**). IF staining further affirmed the nuclear positivity of BRMS1 and P53 in astrocytoma grades (**Figure 4C**), indicating the possible association between *mut*P53 and BRMS1 in astrocytoma **(Supplementary Table S2)**. The Kaplan-Meier curve showed poor survival in patients with positive BRMS1 and P53 expression **(Figure 4D)**. The log-rank (Mantle-Cox) test showed significant survival distribution *(*P≤0.0008)* between the mentioned groups with hazard ratio 5.6 (95% CI 2.062 to 15.71).

3.3.4 Positive correlation between BRMS1 and EMT proteins in malignant astrocytoma

Earlier report has demonstrated the pathological connection between BRMS1 and EMT in lung carcinoma (Liu et al. 2015). In the previous chapter, we unveil the presence of EMT in malignant glioma that inflicts glioma malignancy and chemoresistance in the support of recent reports (Du et al. 2017; Iwadate 2016; Wang et al. 2015).

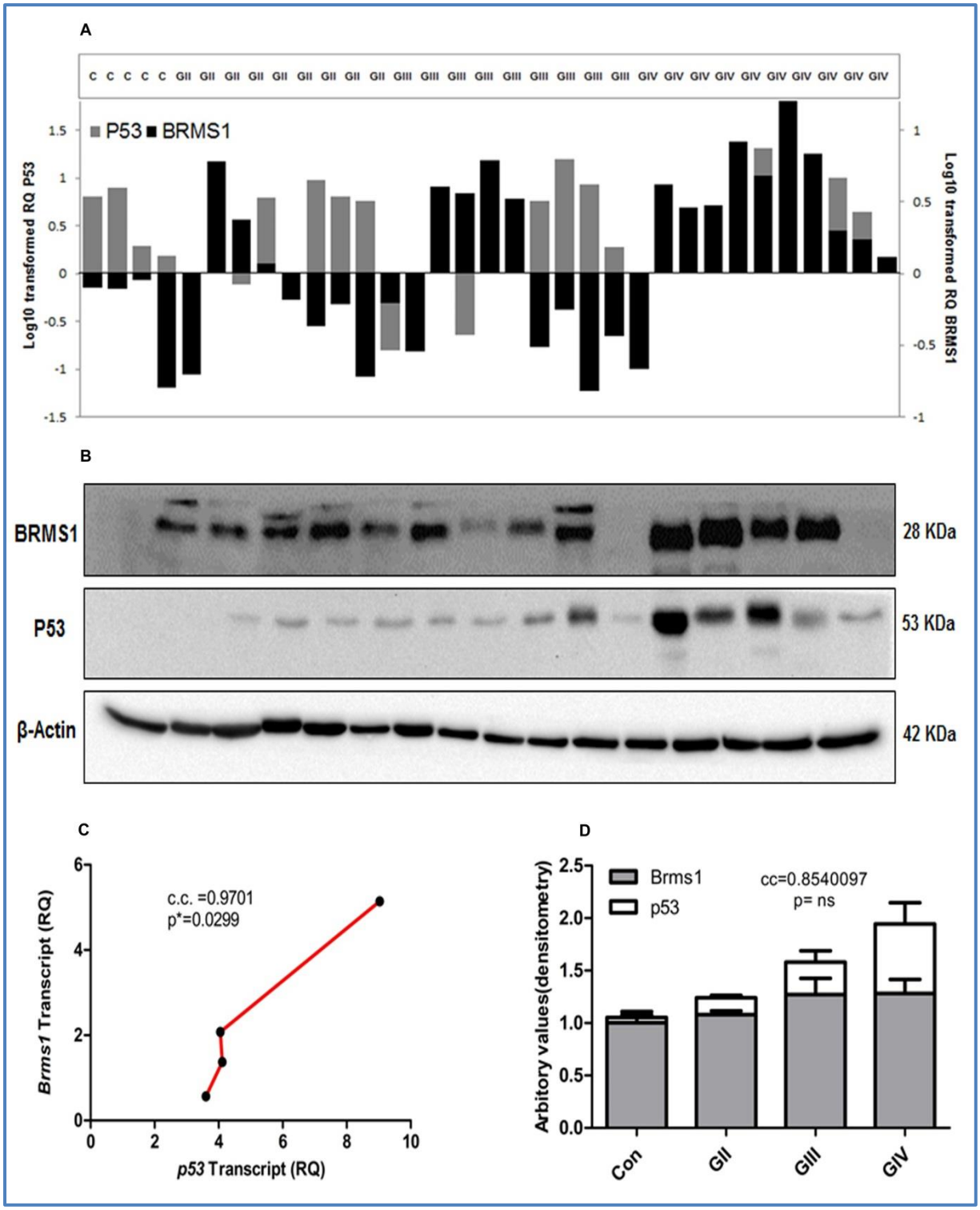

Figure 3. The positive association between *BRMS1* and *mut*P53. **(A)** The transcript and **(B)** protein expression analysis of P53 by qPCR and western blots show a positive expression pattern in BRMS1 expressing tissues. **(C, D)** The correlation study reveals a significant positive correlation between BRMS1 and *mut*P53 at mRNA and protein levels.

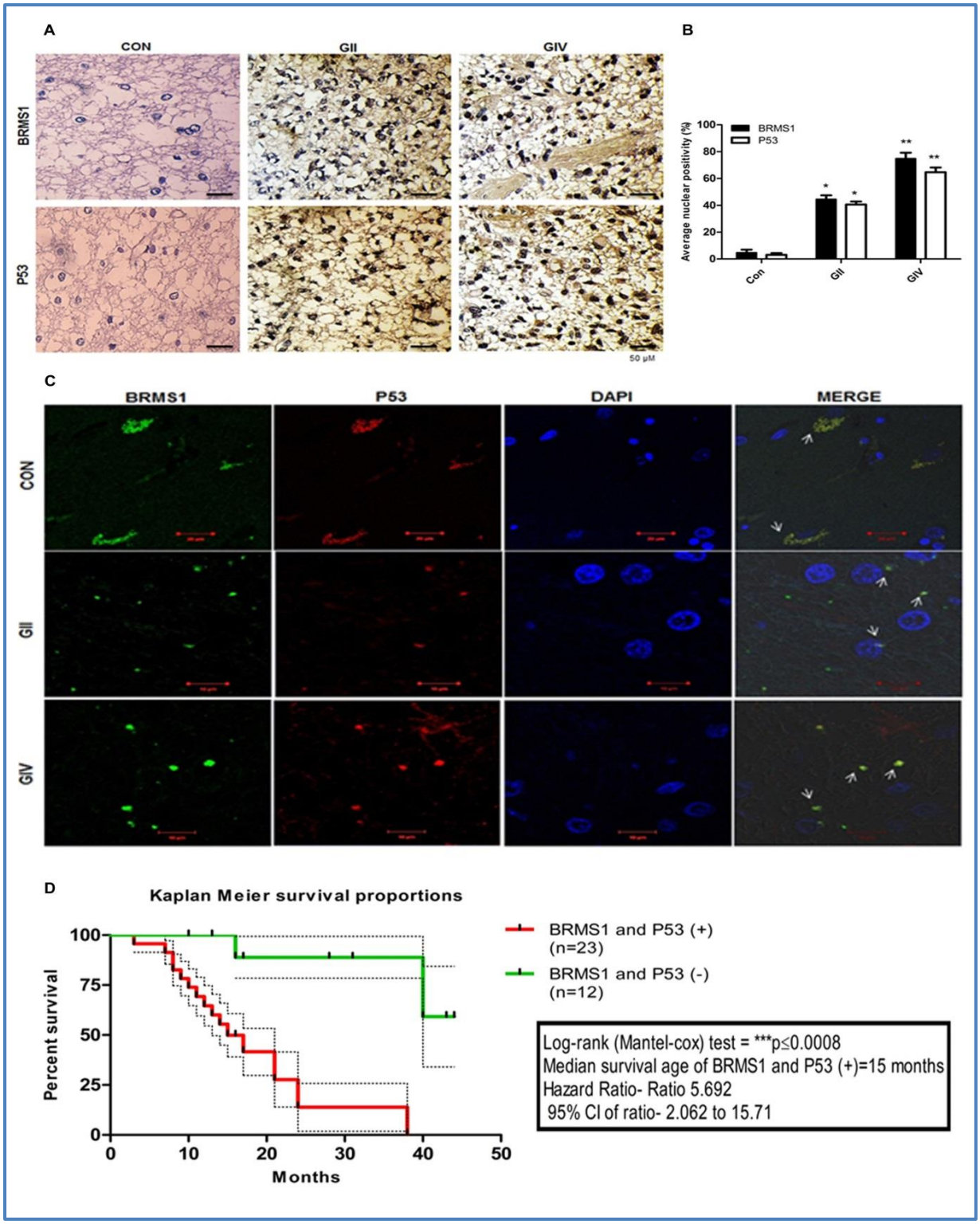

Figure 4. BRMS1 and P53 nuclear positivity may worsen astrocytoma malignancy and patient poor survival. (A, B) The IHC images depict significantly high BRMS1 and P53 nuclear positivity in GIV tissue as compared to the GII and control tissue. **(C)** IF images confirm nuclear positivity of BRMS1 and P53 in astrocytoma grades **(D)** The Kaplan-Meier graph indicates BRMS1 and P53 positive patients show dismal survivability, Log-rank test reveals significant survival difference ($p \leq 0.0008$) with hazard ratio 5.6 (95% CI2 062 to 15.71). The bar represents SEM and p-value ≤ 0.05 was considered as statistically significant.

In view of these facts to determine the etiological association between BRMS1 and EMT in astrocytoma malignancy, we checked the expression pattern of BRMS1 and EMT protein in astrocytoma grades. The immunoblot **(Figure 5A)** results showed the positive correlation between BRMS1 and EMT proteins in astrocytoma grades as compared to the control; summarized in **Table 2**. Interestingly in malignant astrocytoma, the BRMS1 expression pattern was similar to the expression pattern of EMT protein vimentin and β-catenin **(Figure 5A)**. The correlation study illustrates the positive correlation between BRMS1 and vimentin r^2= 0.9508; p=0.049 **(Figure 5B)** and BRMS1 and β-catenin r^2= 0.8864; p=0.188 **(Figure 5D)** indicating the plausible association between BRMS1 and EMT proteins in astrocytoma malignancy.

Table 2: Association between BRMS1 and EMT associated proteins in different grades of astrocytoma

Tissues	Con (n=6)	GII (n=8)	GIII (n=8)	GIV (n=10)	Total (n=26)
BRMS1 (+)	01 (16.66%)	04 (50%)	05 (62.5%)	08 (80%)	17 (65.38%)
Vimentin (+)	00	05 (62.5%)	05 (62.5%)	07 (70%)	17 (65.38%)
β-catenin (+)	4 (66.66%)	05 (62.5)	06 (75%)	09 (90%)	20 (76.92%)

(+) depict positive expression, Control (Con), Grade II (GII), Grade III (GIII), Grade IV (GIV)

3.3.5 Increased BRMS1 expression in *mut*P53 GBM cell lines

We also checked the BRMS1 expression in multiple GBM cell lines such as U373, LN18 and T98 (*mut*P53) and U87 (*wt*P53 cell line). The *in-vitro* expression analysis revealed a differential expression pattern of BRMS1 and P53 at mRNA and protein levels **(Figure 6A, B)**. Interestingly, we observed the increased BRMS1 and P53 expression in U373, LN18 and T98 cell lines as compared to U87 **(Figure 6A, B)**. U87 a *wt*P53 cell line showed low BRMS1 and very low P53 protein expression because of short half-life of P53 protein (Van Meir et al. 1994).

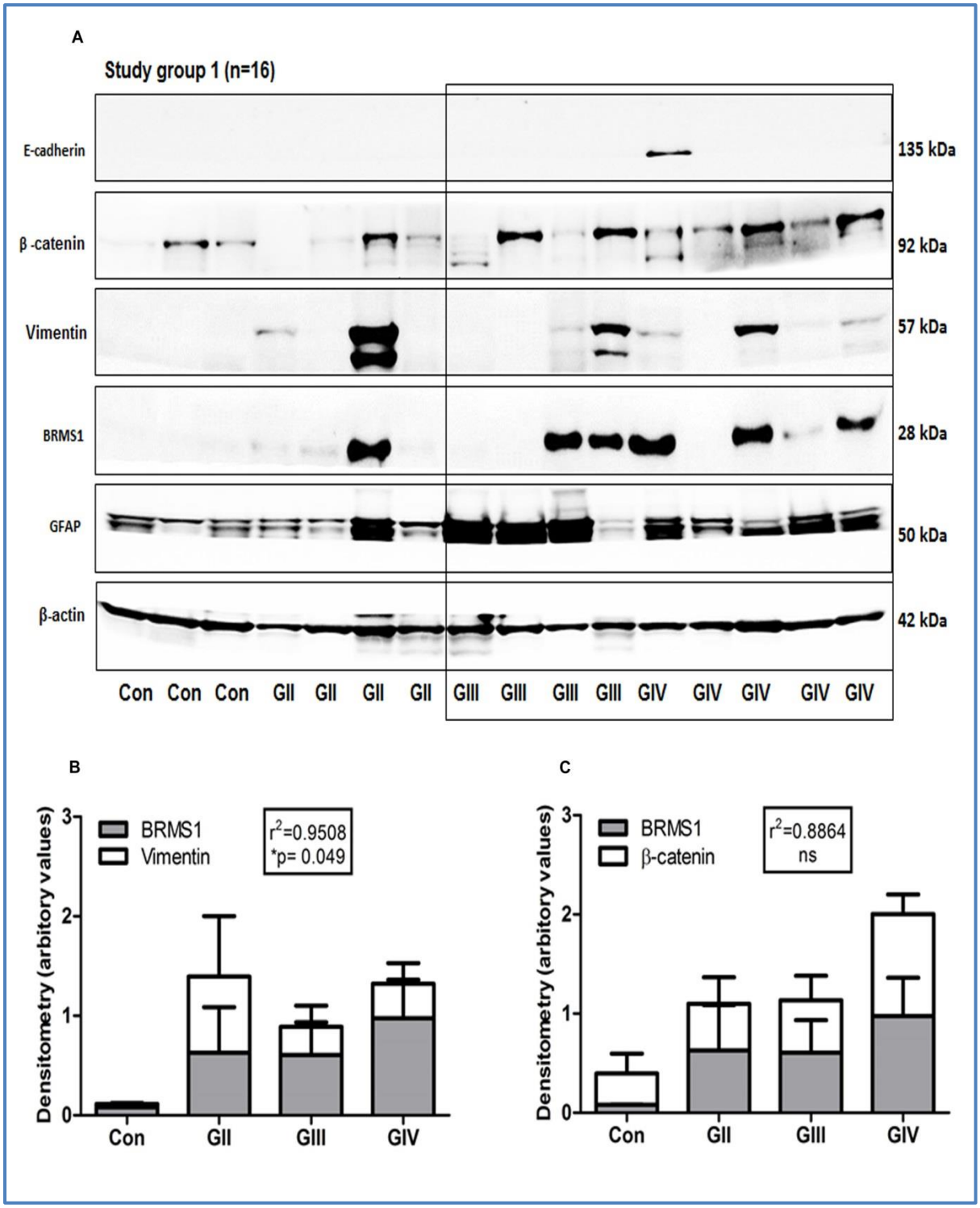

Figure 5. The positive association between BRMS1 and EMT proteins. (A) The expression analysis of BRMS1 and EMT associated proteins by western blots shows positive expression patterns between BRMS1 and EMT proteins in astrocytoma tissues. **(C, D)** The correlation study reveals a positive correlation between BRMS1 and vimentin and BRMS1 and β-catenin.

These results were consistent with the NCI-60 transcript online database (http://discover.nci.nih.gov/cellminer/); **(Figure 6C)**, widely used in cancer research (Weinstein 2006), wherein we have analyzed the transcript expression of *BRMS1* in various cancer cell lines with both *mut*P53 and *wt*P53 genetic composition.

3.3.6 BRMS1 knockdown significantly reduced cell growth in LN18 cell line

Further, we performed *BRMS1* siRNA transfection study to verify BRMS1 involvement in GBM cell growth and colonization. BRMS1 knockdown was confirmed at transcript and protein levels in our experimental sets in both cell lines **(Figure 6D)**. The phase contrast microscopic images showed altered cell morphology of U87 and LN18 cells in terms of cell size and shape upon *BRMS1* siRNA transfection compared to the NT siRNA and normal control **(Figure 6E)**. MTT results revealed BRMS1 silencing inhibits cell proliferation of U87 and LN18 after 48 hours of BRMS1 siRNA treatment in comparison to the NT siRNA and control **(Figure 6F, G)**. However, the significant inhibition of cell proliferation was observed only in LN18 cell line *(*P≤0.007)* **(Figure 6G)**.

3.3.7 BRMS1 silencing sensitized apoptosis and reduced colony forming efficiency of GBM cells

To demonstrate whether BRMS1 silencing imparts cell growth and apoptosis in GBM cell lines, we checked the status of apoptotic protein upon BRMS1 knockdown in U87 and LN18 cell lines. The western blotting (**Figure 7A)** showed an increased bax and caspase 3 expressions together with PARP suggesting that BRMS1 silencing may sensitize apoptosis. However, as shown in **Figure 7A, C** LN18 cells displayed significant increase of apoptotic proteins compared to the U87 (**Figure 7A, B).**

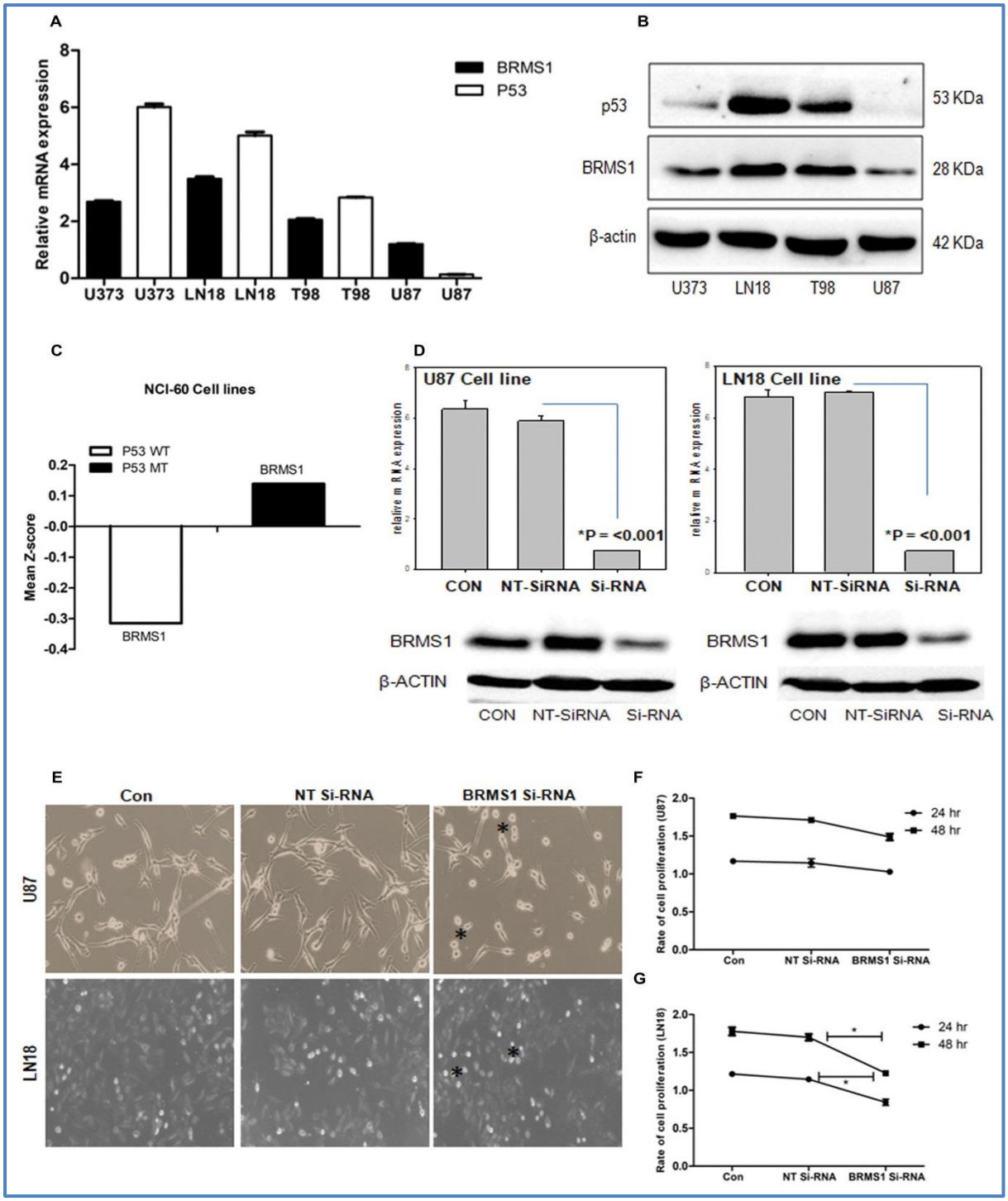

Figure 6. High BRMS1 expressions in *mutP53* GBM cell lines; reduces cell growth upon BRMS1 knockdown. (A) The graph shows mRNA expression pattern of BRMS1 and P53 in GBM cell lines **(B)** Western blots represents a high BRMS1 expression in *mut*P53GBM cell lines. **(C)** The NCI-60 transcript analysis indicates a high *BRMS1* mRNA level in *mut*P53 cell lines compared to the *wt*P53 cell lines. **(D)** The significant *BRMS1* inhibition was confirmed at mRNA and protein level upon *BRMS1* siRNA transfection in U87 and LN18 cell lines. **(E)** Phase contrast images show altered cell morphology in *BRMS1* siRNA treated cell lines. **(F, G)** Graphs reveal significant inhibition of cell proliferation upon *BRMS1* silencing in *mut*P53 LN18 cell line. The p-value ≤ 0.05 was considered as statistically significant.

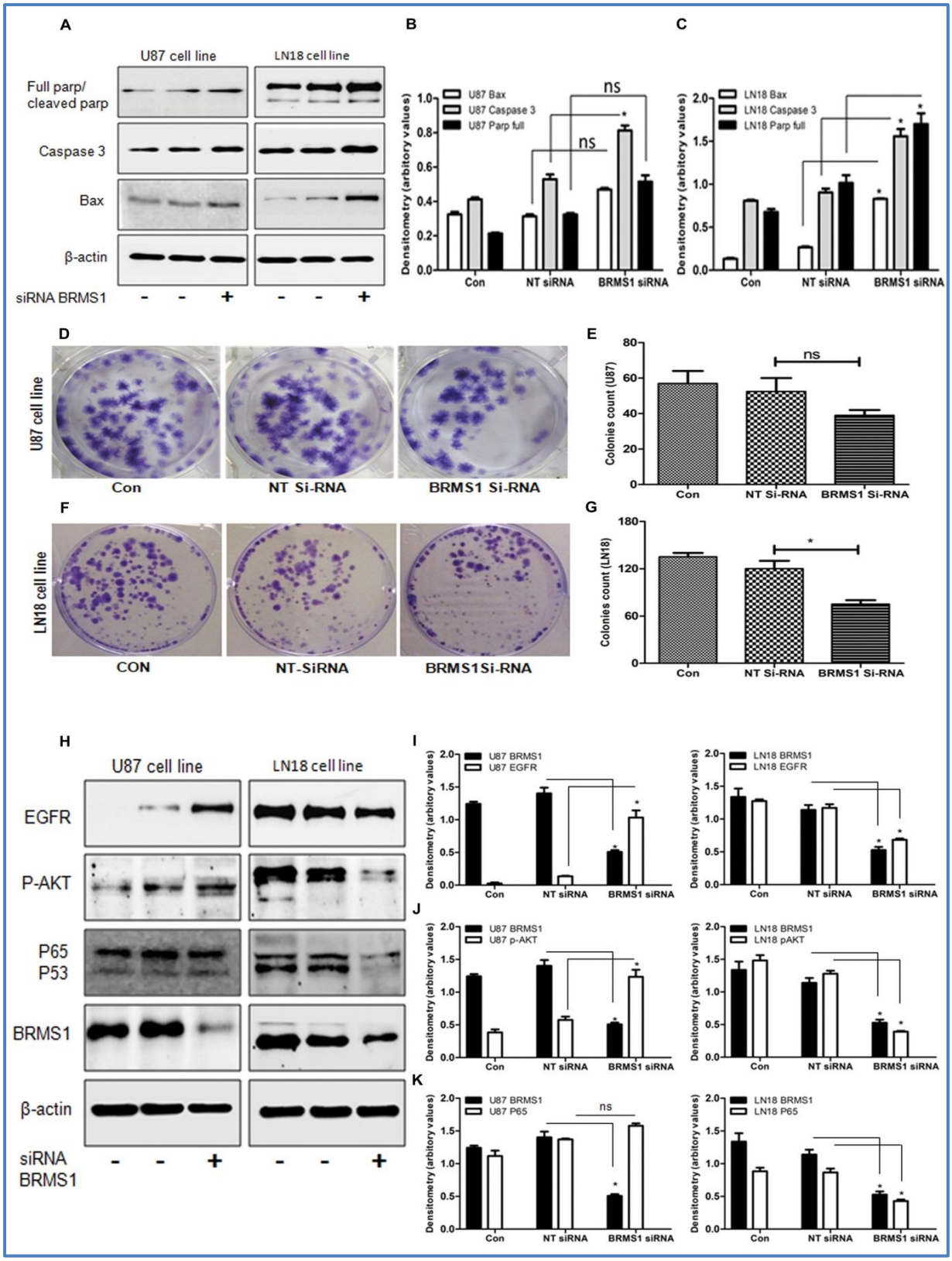

Figure 7. BRMS1 knockdown induces apoptotic proteins and significantly suppress colony formation and EGFR-AKT signaling in LN18 cell line. (A) Western blots and **(B, C)** densitometry graph shows an increased PARP, caspase3 and bax levels in BRMS1 siRNA treated cell lines. **(D, F)** Clonogenic images depict reduced colony formation in both the cell lines after *BRMS1* knockdown.

(E, G) The graph indicates a significant reduction of colony formation in LN18 cell line. **(H)** Western blots and **(I-K)** densitometry graphs represent selective induction or inhibition of EGFR, pAKT, and NF-ϰB-p65 upon BRMS1 silencing. At least three independent experiments were performed in triplicates; the bar represents SEM and p-value ≤ 0.05 was considered as statistically significant.

The clonogenic assay also evidenced less number of colonies in U87 **(Figure 7D)** and LN18 **(Figure 7F)** cell lines upon BRMS1 siRNA treatment in comparison to the NT siRNA and control. Though, the reduction in colony formation was statistically significant only in LN18 cell line **(Figure 7E, G).**

3.3.8 BRMS1 knockdown selectively modulated EGFR-AKT and EMT signaling

EGFR plays a crucial role in cell survival and apoptosis via PI3K-AKT signaling (Azuaje et al. 2015). Here, we have also evaluated the EGFR and pAKT expression levels in BRMS1 siRNA and NT siRNA treated GBM cells. The western blots showed an increased EGFR and pAKT expression in U87 (*wt*P53) cells while in LN18 (*mut*P53) cells EGFR, pAKT and NF-ϰB-p65 expressions were decreased upon BRMS1 knockdown **(Figure 7H)**. The densitometry quantification further revealed significant increased or decreased expression of EGFR, pAKT and NF-ϰB-p65 upon BRMS1 knockdown **(Figure 7I-K)** which is in agreement with previous studies that suggest the cell specific signaling crosstalk of BRMS1 (Kelly et al. 2005; Vaidya et al. 2008). Further, to demonstrate the molecular association of BRMS1 and EMT in GBM we checked EMT proteins upon BRMS1 silencing. The western blots show significant inhibition of vimentin, β-catenin and snail along with NF-ϰB proteins in LN18 cell line **(Figure 8A)** while in U87 cells we did not find significant inhibition of EMT proteins nor NF-ϰB proteins **(Figure 8A)**, indicating the peculiar molecular mechanism of BRMS1 in an individual cell line. We also proposed plausible signaling cascade of EMT suppression upon BRMS1 knockdown **(Figure 8A)**.

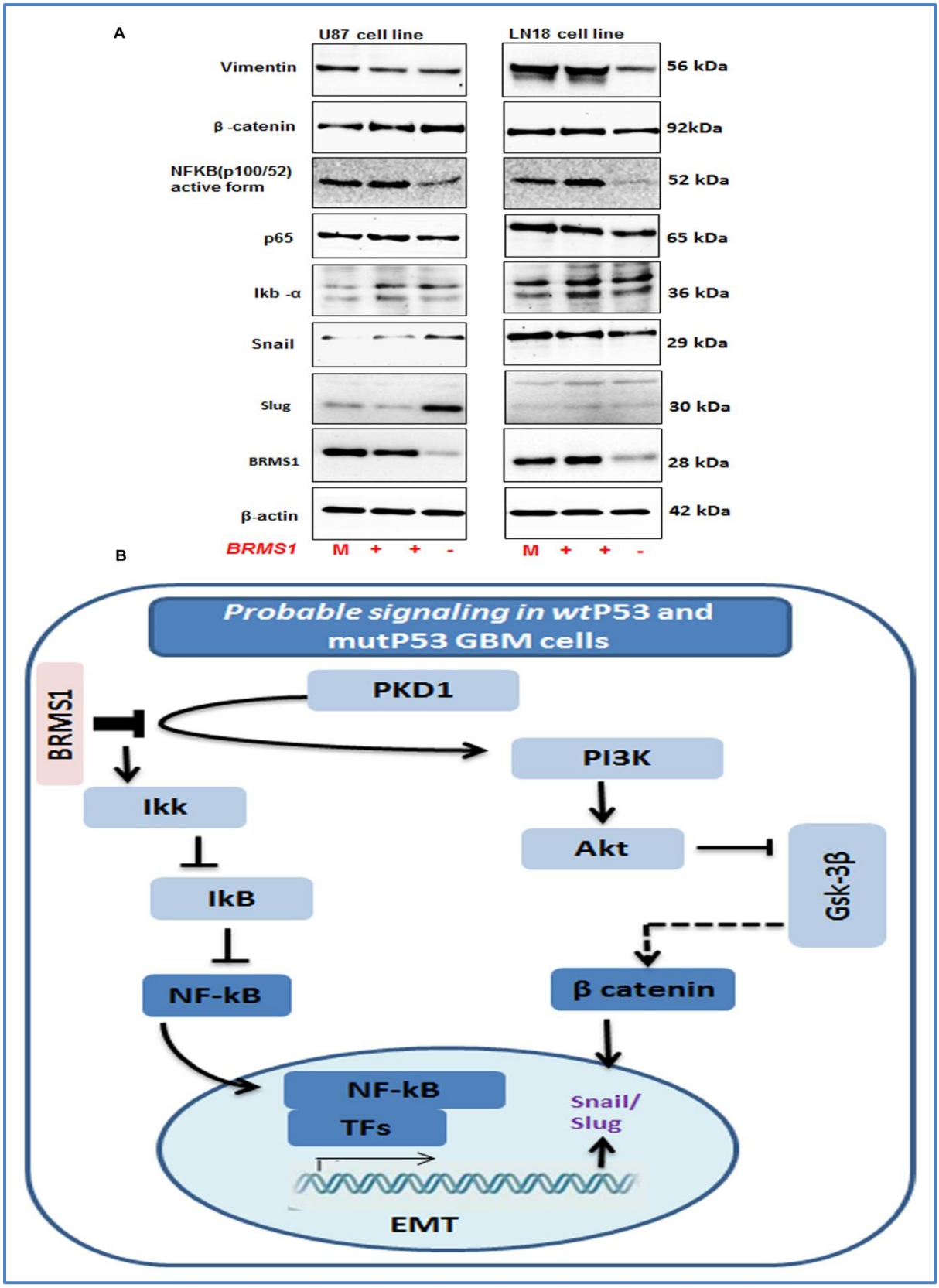

Figure 8. BRMS1 knockdown inhibits EMT signaling in LN18 cell line. (A) Western blots show decrease vimentin, β-catenin, snail and NF-κB proteins in BRMS1 siRNA treated LN18 cell line. **(B)** Plausible signaling cascade of EMT inhibition upon BRMS1 silencing.

3.3.9 BRMS1 silencing attenuate cell migration and/or invasion and sensitized TMZ resistance in LN18 cells

EMT in cancer cells enhanced cellular invasiveness and resistance to apoptosis and drug treatment (Thiery 2002). In the current objective, we have already shown a positive association of BRMS1 with EMT proteins and the *in-vitro* study disclosed BRMS1 silencing suppresses EMT signaling. In this context to further evaluate the BRMS1 potentials, we tested invasive ability and TMZ resistance of GBM cells upon BRMS1 knockdown. Transwell invasion assay showed reduced cell invasion and migration in both cell lines upon BRMS1 siRNA treatment as compared to the control and NT siRNA **(Figure 9A)**. The statistical analysis revealed a significant reduction only in LN18 cell line **(Figure 9B, C)**. MTT result displayed a non-significant cell growth inhibition in combined BRMS1 siRNA+ TMZ treatment as compared to the BRMS1 siRNA and TMZ (100μM) in U87 cell line **(Figure 9D)**. While in LN18 cell lines which a TMZ resistance cell line (Happold et al. 2012), we observed a significant cell growth inhibition in combined BRMS1 siRNA+ TMZ treatment as compared to the BRMS1 siRNA and TMZ (100μM) **(Figure 9E)**, this confirm the therapeutic significance of BRMS1 by repressing EMT and TMZ resistance.

3.3.10 The *BRMS1* transcript is positively correlated with *uPA* and negatively correlated with *ING4* in astrocytoma grades

Earlier investigations have shown that BRMS1 inhibits uPA and upregulate ING4 thereby suppressing tumor malignancy (Cicek et al. 2009; Li and Li 2010). We segregated BRMS1 expressing astrocytoma tissues and checked the transcript expressions of uPA and ING4 and correlated with *BRMS1* expression.

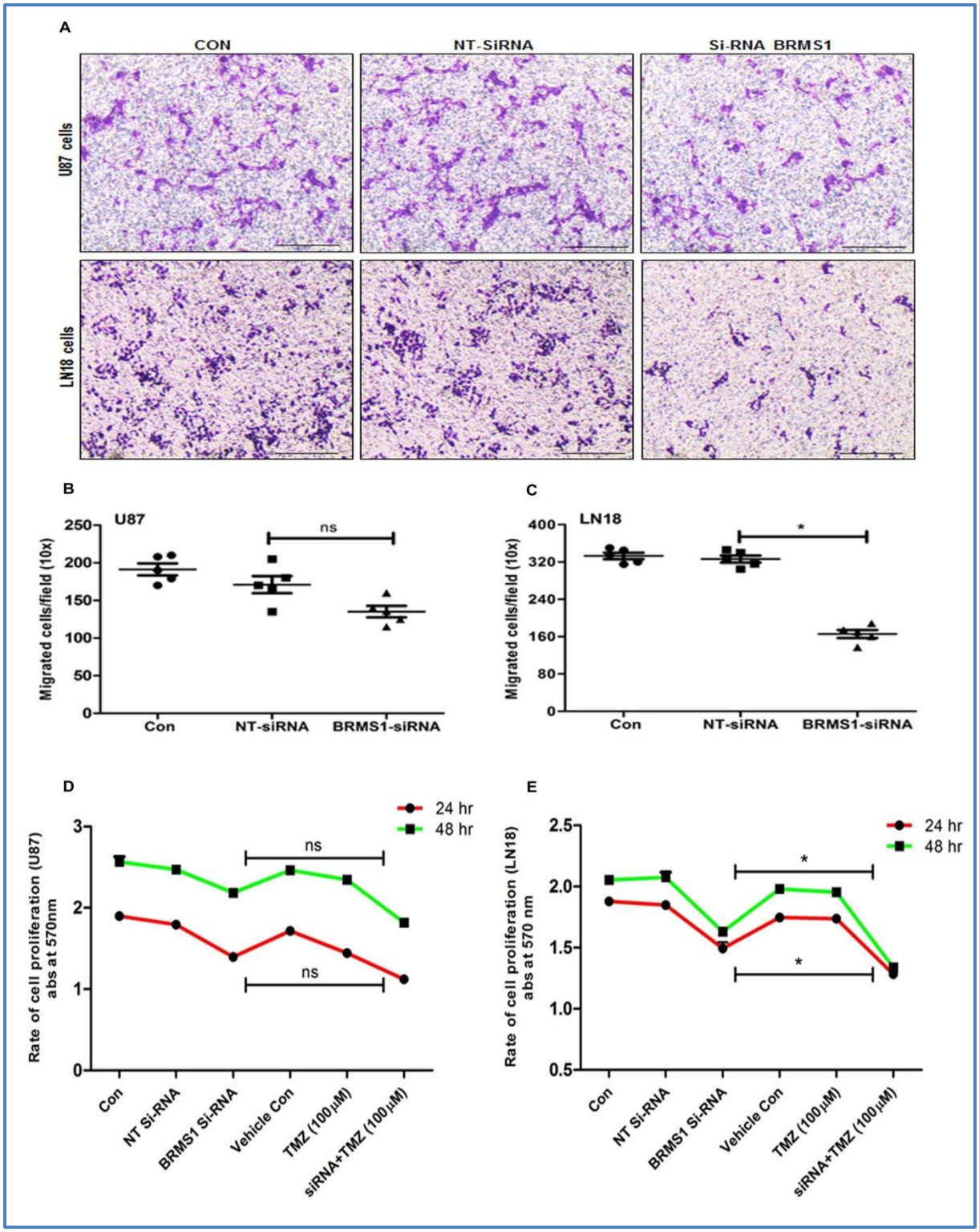

Figure 9. BRMS1 silencing reduced cell invasion and sensitized TMZ resistance in LN18 cells. (A-C) Transwell invasion assay shows reduce cell invasion upon BRMS1 silencing in both the cell lines but significant reduction is observed only in LN18 cell line. **(D)** MTT results displays non-significant cell growth inhibition in combined BRMS1 siRNA+ TMZ treatment as compared to the BRMS1 siRNA and TMZ (100μM) in U87 cell line. (**E)** While in LN18 cell line significant cell growth inhibition is observed in combined BRMS1 siRNA+ TMZ treatment as compared to the BRMS1 siRNA and TMZ (100μM).

Our results revealed that the BRMS1 expressing samples showed high mRNA expression of *uPA* and low mRNA expression of *ING4* **(Figure 10A, B)**. The correlation analysis disclosed positive *BRMS1* mRNA expression with *uPA* (r^2= 1.0; p≤ 0.083) **(Figure 10A)** and negative correlation with *ING4* mRNA levels (r^2= -0.040; p≤ 0.75) **(Figure 10B)**. Further, *BRMS1* knockdown showed mRNA inhibition of *uPA* and *ING4* in both the cell lines by PCR **(Figure 10C)**, consistent with NCI-60 transcript analysis that showed positive expression of *uPA* with *BRMS1* in *mut*P53 cell lines, while *ING4* showed negative expression with *BRMS1* irrespective of P53 status **(Figure 10D)**. Furthermore, the qPCR results also showed significant inhibition of BRMS1 downstream targets uPA and ING4 **(Figure 10E, F)**.

3.4 Discussion

Cancer metastasis is the major threat of cancer related deaths. In the last couple of years, *BRMS1* was well studied in various cancers and recognized as a potential metastasis suppressor gene (Samant et al. 2000; Welch et al. 2016). In the present study, we have demonstrated the BRMS1 expression and its clinical and functional relevance in astrocytoma progression and malignancy. The *BRMS1* transcript and protein expression analysis signify an upregulated expression in high grade (GIII+GIV) astrocytoma tissue compared to the low grade (GII) and control tissue. BRMS1 upregulation was associated with poor prognosis in GBM and significantly correlated with grade progression ($p<0.014$) but not with patients age ($p=0.47$) or sex ($p=0.89$) (Table 1). These contrasting observations are in accordance with the previous studies (Hicks et al. 2006; Mei et al. 2014; Zhang et al. 2006). However, recent reports infer BRMS1 overexpression and its association with cancer progression, malignancy and poor prognosis (Bucciarelli et al. 2018; Ventura et al. 2014; Yang et al. 2016) which are in agreement with our study.

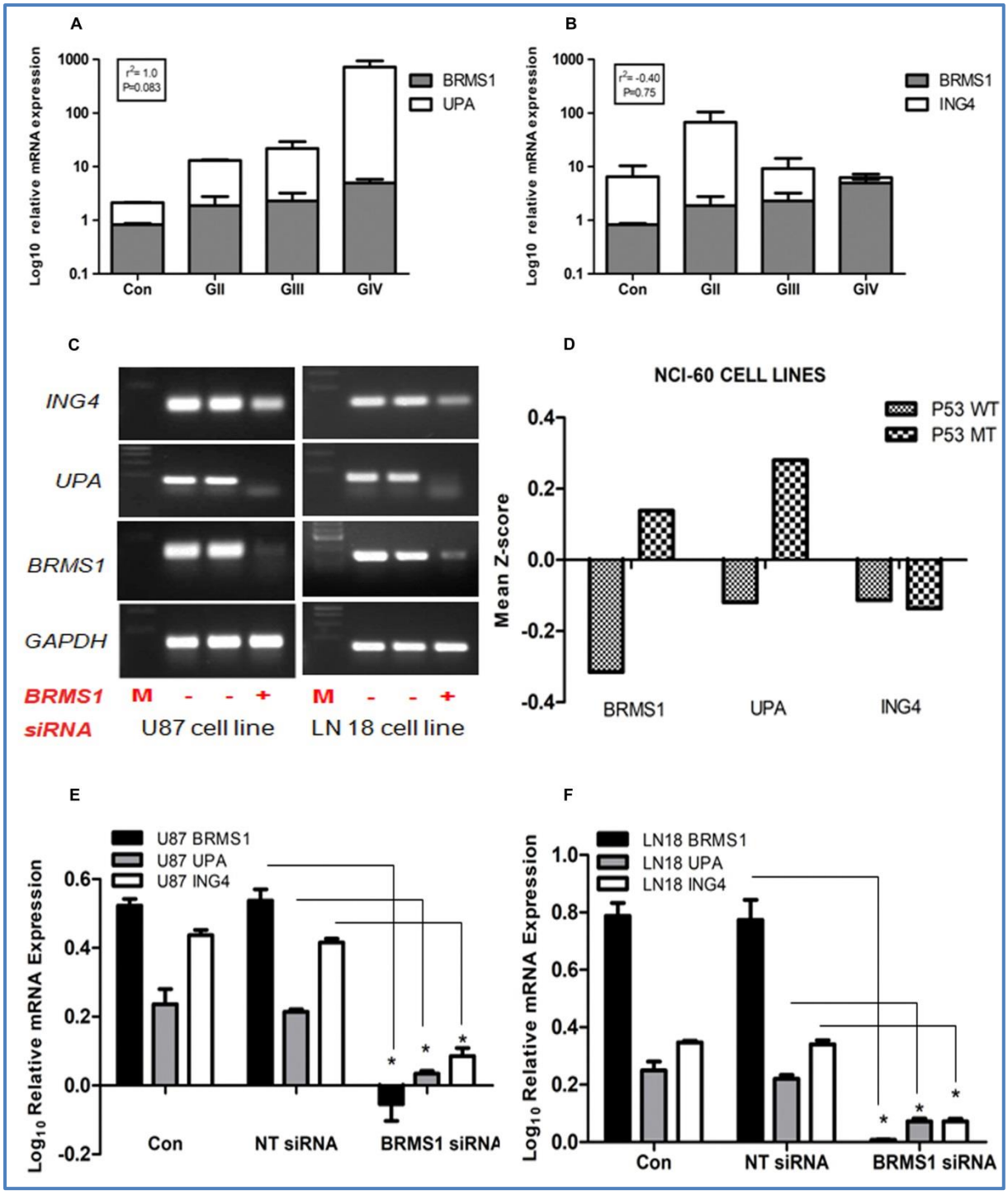

Figure 10. *BRMS1* correlation with its downstream targets in astrocytoma tissues and *mut*P53 GBM cells. (A, B) Graph represents a positive and the negative mRNA expression correlation between uPA (r^2= 1.0; p≤ 0.083) and *ING4* (r^2= -0.040; p≤ 0.75) with *BRMS1* respectively in astrocytoma grades. **(C)** Agarose gel images show significant transcript inhibition of *uPA* and *ING4* upon *BRMS1* knockdown in both cell lines. **(D)** NCI-60 data confer positive mRNA expression pattern of *uPA* with *BRMS1*, while *ING4* show a negative mRNA expression pattern with *BRMS1* in *mt*P53 cell lines. **(E, F)** QPCR graphs affirm significant transcript inhibition of *uPA* and *ING4* upon *BRMS1* knockdown**.** The p-value ≤ 0.05 was considered as statistically significant.

Earlier reports described that the BRMS1 suppresses tumor metastasis by affecting critical steps involved in cancer metastasis, such as cell migration, invasion, angiogenesis, apoptosis and cytoskeleton rearrangement (Kodura and Souchelnytskyi 2015; Li and Li 2010; Mei et al. 2014; Phadke et al. 2008). BRMS1 inhibition in a cell with oncogenic driver mutation like P53 loss and K-Ras mutation induces cell migration and invasion (Hall et al. 2014). Recent study described that BRMS1 can suppress cancer malignancy and invasion by modulating EMT (Liu et al. 2015). P53 is a frequently reported driver mutation which can regulate various signaling pathways such as NF-κB, Pi-3K-AKT, EMT etc. that are involved in GBM progression or malignancy (Fulci et al. 1998; Noroxe et al. 2016; Wang et al. 2013). Suggesting, it is quite meaningful to decipher the association between BRMS1 and P53 along with EMT in astrocytoma grades. Here we showed the high P53 transcript and protein levels in BRMS1 expressing astrocytoma tissues compared to the control and low grade tissue. Also, the NCI 60 cell line data base evidenced increased BRMS1 expression in *mut*P53 cell lines compared to the *wt*P53 cell lines. The correlation analysis revealed a positive expression correlation of *mut*P53 and EMT associated proteins with BRMS1 in astrocytoma grades, which may confer dismal prognosis, suggesting a plausible etiological association of BRMS1 with *mut*P53 and EMT in astrocytoma malignancy as shown in other cancers (Chen et al. 2016; Hall et al. 2014; Liu et al. 2015).

Further, to support the clinical outcome, we performed *in-vitro* study and demonstrated the differential expression of BRMS1 in GBM cell lines. Interestingly, the cell lines with *mut*P53 (U373, LN18 and T98) showed increased *BRMS1* transcript and protein expression in comparison to *wt*P53 cell line (U87) corroborating with earlier reports which explained the cell specific expression and activity of BRMS1 (Cook et al. 2012; Kelly et al. 2005). The BRMS1 knockdown altered cell morphology with restricted cell growth and colony formation.

The plausible cause of inhibited cell growth may be apoptosis sensitization and/or modulated EGFR-AKT and EMT signaling upon BRMS1 knockdown. We also evaluated GBM invasion and chemoresistance upon BRMS1 knockdown to establish the connection between BRMS1 and EMT in GBM. Our data showed reduced GBM cell invasion and TMZ resistance upon BRMS1 silencing demonstrating the relationship between BRMS1 and EMT. However, this was quite surprising that the BRMS1 silencing in two cell lines with *mut*P53 and *wt*P53 showed dissimilar effects in the notion of previous reports (Cook et al. 2012; Kelly et al. 2005; Vaidya et al. 2008).

Furthermore, the BRMS1 downstream proteins such as *uPA*, *OPN* and *ING4* are supposed to be instrumental in *BRMS1* regulated metastasis suppression (Cicek et al. 2009; Cicek et al. 2005; Li and Li 2010; Wu et al. 2012). In brain tumors, the altered *uPA* and *ING4* expression served as a prognostic and metastasis biomarkers (Kit et al. 2017; Klironomos et al. 2010). In the current study, we showed the positive and a negative mRNA correlation of *uPA* and *ING4* respectively with *BRMS1* in astrocytoma grades and GBM cell lines. The *in-vitro* data revealed significant inhibition of uPA and *ING4* transcript after *BRMS1* knockdown. In fact, the NCI-60 cell lines data also followed a similar expression pattern, particularly in *mut*P53 cell lines suggesting BRMS1 role in GBM progression. Though we demonstrated the clinical and molecular significance of BRMS1 in astrocytoma progression and its concomitant signaling in *wt* and/or *mut*P53 GBM, the *in-vivo* studies could further justify the BRMS1 involvement in the *mut*P53 GBM progression and malignancy.

3.5 Conclusion

In summary, we report an upregulated BRMS1 expression and its association with GBM malignancy and poor prognosis. Interestingly, we also observed the positive relationship of BRMS1 with *mut*P53 and EMT in GBM may worsen disease malignancy and patient's poor survivability. The *in-vitro* study showed reduced cell growth, cell survivability, cell invasiveness and TMZ resistance via modulating EGFR-AKT and EMT signaling upon BRMS1 knockdown in *mut*P53 cells, suggesting BRMS1 can be a predictive and therapeutic candidate for *mut*P53 GBM.

Additional Information

Study Approval

All procedures performed in studies involving human participants were approved by Institutional ethics committee (IEC), University of Hyderabad, Hyderabad (TS), with IEC reference number UH/IEC/2016/18. The written informed consent was obtained from all participants included in the study.

Supporting Information

Supplementary Tables

Table S1

Table S1: Real time PCR primer sequences		
S.No.	**Primer name**	**Primer sequences (5'-3')**
1	*BRMS1* Forward primer	GCT CCG AGA TGG ATG ATG AGG
	BRMS1 Reverse primer	AAT CTT GAG GCT CCG CTG CAG
2	*P53* Forward primer	AGGTTGGCTCTGACTGTACC
	P53 Reverse primer	CACCTCAAAGCTGTTCCGTC
3	*UPA* Forward primer	GCC TTG CTG AAG ATC CGT TC
	UPA Reverse primer	GAT CGT TAT ACA TCG AGG GGC A
4	*ING4* Forward primer	TCG TGC GCA CAA GTC CTG AG
	ING4 Reverse primer	CCA CTC AAT GGA ACA ATC AGG G
5	*GAPDH* Forward primer	AAG GCT GGG GCT CAT TTG CAG
	GAPDH Reverse primer	GCA GGA GGC ATT GCT GAT GAT C

Table S2

Table S2: Association between BRMS1 and mutP53 in astrocytoma (n=48)			
	BRMS1 expression profile		P value
	(+)	(-)	
P53 (+)	39.58% (19)	29.16% (14)	*p=0.045
P53 (-)	27.08% (13)	04.16% (02)	
The association between BRMS1 and P53 were analyzed in astrocytoma tissue (n=48) by Fischer's exact test. Positive or negative expression depicted by (+)/ (-).			

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Chapter 4

Discussion and Summary

Discussion

The conventional concept of tumor or cancer initiation and its establishment is based on genetic alterations that lead to the abnormal expression and function of a gene (Fearon and Vogelstein 1990; Vogelstein et al. 2013), and so far it is believed that a limited numbers of genetic or molecular alterations are pivotal for the rapid proliferation and malignant behaviour of the tumor cells (Folkman et al. 2000; Hanahan and Weinberg 2000). However, with the progress in molecular biology arena it is evident that genetic mutations in the tumors are several and heterogeneous in nature (Folkman et al. 2000; Hanahan and Weinberg 2011). Heterogeneity or ambiguity is a hallmark of malignant cancer attained during the course of cancer progression (Bonavia et al. 2011; Hanahan and Weinberg 2011). It can be characterized by the genetic, epigenetic and/or phenotypic diversity in a tumor. Like other cancer, GBM is highly heterogeneous in nature bearing both genetic and phenotypic variability in a tumor niche (Bonavia et al. 2011; Friedmann-Morvinski 2014).

In GBM the widely accepted concept for the maintenance of heterogeneity is the interaction between intratumoral and tumor microenvironment, and the cancer stem cell model i.e. the stemness property in tumor core region either naïve stem cells such as cancer stem cells (CSCs) and mesenchymal stem cells (MSCs), or acquired during the course of cancer development (epithelial to mesenchymal transition-EMT); which exerts GBM malignancy and resistance against chemo or radiotherapy (Bao et al. 2006; Bonavia et al. 2011; Liu et al. 2006). Indeed, a high degree of heterogeneity at genetic and/or cellular levels endow by unique microenvironment of a tumor imposed establishment and malignancy, and thereby tumor cellular or molecular heterogeneity becomes the major obstacle for the development of successful gene or drug therapies (Bao et al. 2006; Liu et al. 2006).

Numerous studies have elucidated the underlying molecular and cellular events during cancer or GBM progression (Furnari et al. 2007; Hanahan and Weinberg 2000; Liu et al. 2009; Louis et al. 2016). However, the present scenario and upcoming information infers that to develop an effective and successful therapy one has to thoroughly understand the mechanistic mechanism behind the tumor dynamics. Though the presence of EMT like process is recently recognized in glioma, more investigation is needed to delineate its translational relevancies. In the present study, we demonstrated the clinical significance of EMT like process in glioma and its therapeutic relevance through the chemical and molecular approaches.

The expression analysis of EMT associated proteins in glioma tissues revealed negative expression of E-cadherin, differential weak expression of N-cadherin and upregulation of GFAP, vimentin and β-catenin in different grades of glioma as compared to the control tissues. The negativity of E-cadherin and increased expression of GFAP, vimentin and β-catenin is associated with glioma malignancy and dismal prognostic outcomes. Interestingly, the correlation study indicated a positive correlation of GFAP and β-catenin with vimentin and a negative correlation between E-cadherin and vimentin in glioma tissues. These observations support EMT 'all or nothing' event and justify the independency of cadherin switch or noncanonical EMT process in glioma in the notion of previous studies (Iser et al. 2017; Utsuki et al. 2002).

The emerging reports evidenced the therapeutic significance of EMT by PPI - pantoprazole in gastric cancer and BRMS1 in lung carcinoma (Feng et al. 2016; Liu et al. 2015; Zhang et al. 2015), but nothing is known in context to astrocytoma malignancy and chemoresistance. In fact, with the inference of previous studies, we also believe that EMT targeting through a PPI can be a better approach to target glioma. Moreover, PPI a known pH modulator can compromise tumor acidity in addition to EMT.

Our data revealed that rabeprazole treatment inhibits glioma cell growth, altered glioma cell morphology and induced mitochondrial-mediated apoptotic cell death in agreement with previous reports (Canitano et al. 2016; De Milito et al. 2010). We demonstrate rabeprazole administration inhibited cell migration and cell invasion together with EMT suppression by targeting Akt/Gsk-3β/β-catenin signaling.

The *in-vivo* data showed restricted tumor growth and malignancy in the confluence of EMT inhibition representing clinical observations. Further, we demonstrated that the rabeprazole treatment suppresses NF-κB signaling and affirmed with TNF-α induced EMT inhibition by attenuating NF-κB-P65 expression and nuclear translocation, in accordance with previous reports which show TNF-α induced EMT pathway through NF-κB activation (Li et al. 2012; Wang et al. 2013a). Coinciding with the anti-EMT and anticancer efficacy of rabeprazole, we demonstrated the TMZ sensitization by rabeprazole on TMZ resistant cell line, as evident from cell cytotoxic and clonogenic assays. Simultaneously, we also showed the significant inhibition of EMT protein vimentin and β-catenin in a combined dose of rabeprazole and TMZ.

Recent study described that BRMS1 can suppress cancer malignancy and invasion by modulating EMT (Liu et al. 2015). P53 is a frequently reported driver mutation which can regulate various signaling pathways such as NF-κB, Pi-3K-AKT, EMT etc. that are involved in GBM progression or malignancy (Fulci et al. 1998; Noroxe et al. 2016; Wang et al. 2013b). Suggesting, it is meaningful to decipher the clinical and therapeutic significance of BRMS1 in *mut*P53 GBM and its etiological relevancies with EMT in astrocytoma. Our findings revealed overexpression of BRMS1 in malignant astrocytoma associated with pathological grading and patient's poor survivability. We observed the positive correlation between BRMS1 and *mut*P53 and/or EMT proteins in GBM tissues which may extort GBM malignancy and poor prognosis indicating a possible etiological association of BRMS1 with *mut*P53 and, EMT in astrocytoma

malignancy as shown in other cancers (Feng et al. 2016; Hall et al. 2014; Liu et al. 2015).

To support the clinical outcome, we performed *in-vitro* BRMS1 siRNA transfection study which revealed reduced cell growth, cell migration and increased apoptosis in *mut*P53 GBM cell line upon BRMS1 knockdown. Also, The *BRMS1* knockdown suppressed EGFR-AKT and EMT signaling together with NF-κB expression in *mut*P53 cells. Further, we evaluated GBM invasion and chemoresistance upon BRMS1 knockdown to establish the connection between BRMS1 and EMT in GBM. Our data showed reduced GBM cell invasion and TMZ resistance upon BRMS1 silencing demonstrating the relationship between BRMS1 and EMT. However, this was quite surprising that the BRMS1 silencing in two cell lines with *mut*P53 and *wt*P53 showed dissimilar effects in the notion of previous reports (Cook et al. 2012; Kelly et al. 2005; Vaidya et al. 2008).

Furthermore, the BRMS1 downstream proteins such as *uPA*, *OPN* and *ING4* are supposed to be instrumental in *BRMS1* regulated metastasis suppression (Cicek et al. 2009; Cicek et al. 2005; Li and Li 2010; Wu et al. 2012). In brain tumors, the altered *uPA* and *ING4* expression served as a prognostic and metastasis biomarkers (Kit et al. 2017; Klironomos et al. 2010). The transcript profiling of these downstream targets disclosed a positive and negative correlation of *uPA* and *ING4* with *BRMS1* respectively. The *in-vitro* data revealed significant inhibition of uPA and *ING4* transcript after *BRMS1* knockdown. In fact, the NCI-60 cell lines data also followed a similar expression pattern, particularly in *mut*P53 cell lines, suggesting BRMS1 role in GBM progression and chemoresistance advocating its prognostic and therapeutic potentials.

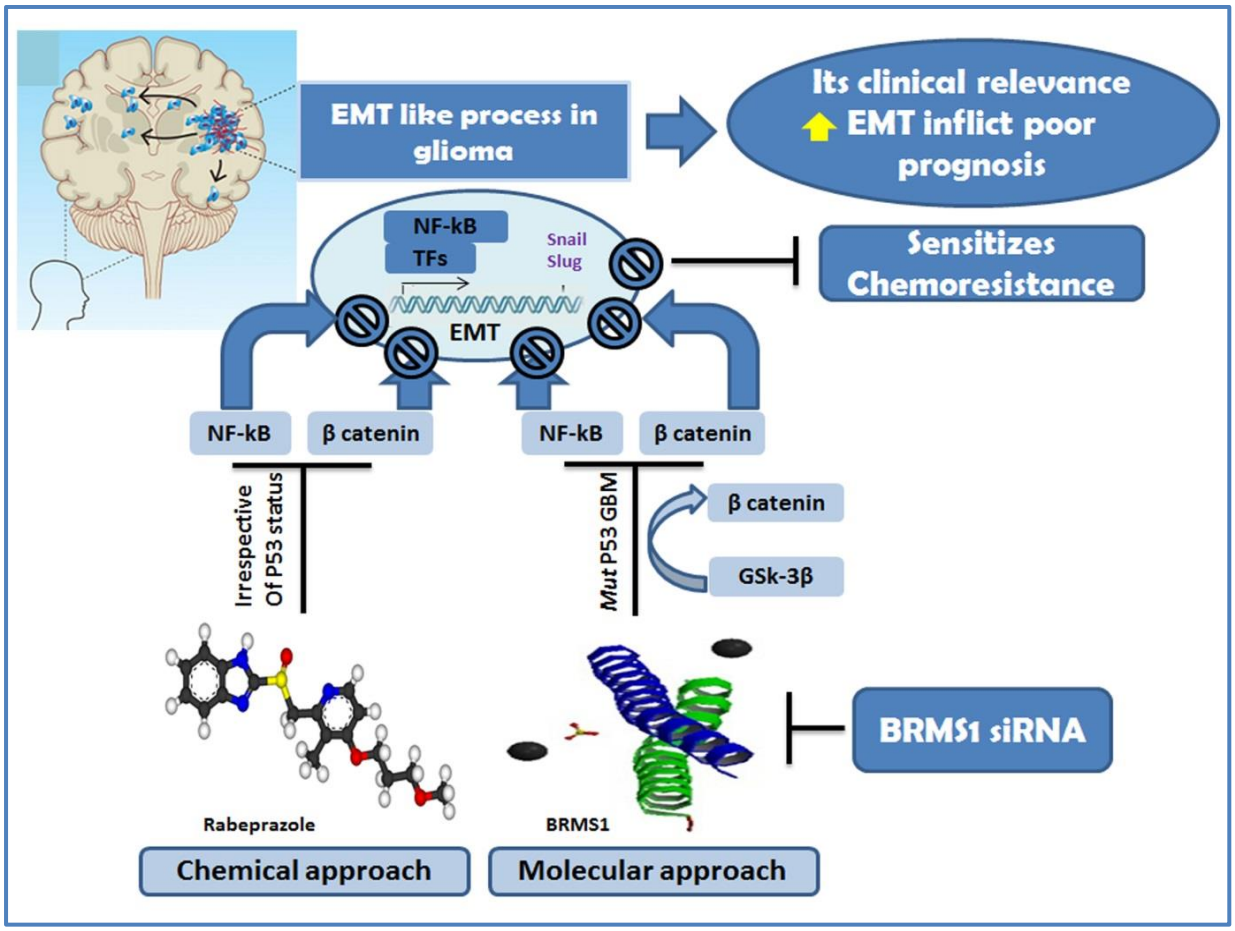

Figure 1. Proposed model of the study. The figure depicts clinical significance of EMT like process in glioma and its therapeutic relevance to rabeprazole and BRMS1.

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Publications

J Neurooncol (2016) 129:383–387
DOI 10.1007/s11060-016-2183-0

LETTER TO THE EDITOR

Brain tumors incidences and a retrospective clinical analysis from a tertiary hospital in India

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Received: 4 February 2016 / Accepted: 9 June 2016 / Published online: 16 June 2016

Introduction

Brain tumor is a collection of abnormally grown cells in brain, contributing total 256,213 new cases reported globally in 2012. In USA, 68,470 brain tumor cases were reported in the year 2015 [1]. Overall, males are reported to be more prone to brain tumors than females with 7.7 males and 5.4 females per 100,000 persons [2]. World Health Organization (WHO) has classified brain tumors into diverse groups based on histopathological features and cellular origin [3]. Among all groups, gliomas appear to be the most common primary brain tumors [1, 3]. Despite of aggressive chemo and radio therapy, the median survival age for malignant gliomas is very low [4].

Cancer databases are actively monitored in developed countries. The categorized studies provides population based information such as median age of detection; most frequently followed therapies, location of tumor and observed symptoms. These data bases can help to study cancer profile in large cohort [5] which in turn could contribute for better therapeutic measures.

Cancer registry in India is maintained at tertiary levels. However, National cancer registry program was undertaken by Indian Council of Medical Research (ICMR) for registration and epidemiological studies of cancer [6]. In 2008, a study from India reported by Jalali R. and Datta D. conclude that the rates of benign tumor are low in comparisons to malignant tumor in Indian population. This study also suggest the lower median age of patients in Indian population as compared to western countries [7]. The statistical studies in developing countries like India are underscored owing to limited access to the biological data and inefficient registry system.

In the present investigation, we have included 1232 patients and attempted to record clinico pathological information from hospital which includes genders, median age of patients and different types of cell specific brain tumor in a cohort. In addition, we have analyzed the initial symptoms associated with the astrocytoma. This type of statistical information might be useful in studying demographic distribution of brain tumors in both sexes of Indian population.

Methods and results

A directory of patients was maintained with clinicopathological parameters. Each patient was assigned a unique IP number which was used for further reference. Clinical updates were maintained in directory with background information of symptoms, date of surgery, date of radiation and chemotherapy followed by pathological reports. Grades of different types of brain tumors were confirmed from tissue based staining in pathology department. At the end, data was categorized in different tumor types with selected age group.

In this study, total 1232 cases were analyzed for the period of 6 years (January 2009 to December 2014). Upon histopathological reports tumors were categorized in different types such as astrocytoma (subcategorized to four grades as per WHO classification), meningioma, schwannoma,

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Table 1 Statistics of distribution of central nervous system tumors and comparison with data from developed countries

Tumor type	Total cases	Males	Females	<10 years	11–30 years	31–60 years	>60 years	Median age[a]	Median age[b]
Glioma	610								
Pilocytic astrocytoma	51	33	18	12	31	8	0	15.5	23
Diffuse astrocytoma	231	124	107	8	66	149	8	54	33
Anaplastic astrocytoma	88	51	37	3	21	62	2	45	49
Glioblastoma multiformae	203	125	78	4	42	120	37	37.5	62
Unspecified glioma cases	37	22	15	2	10	17	8	–	–
Meningioma	267	79	188	00	33	214	20	43.5	55
GI	231	64	167	00	28	180	23	–	–
GII	27	12	15	00	3	20	4	–	–
GIII	2	1	1	00	00	02	00	–	–
Unspecified cases	7	2	5	00	2	05	00	–	–
Schwannoma	216	89	127	1	40	142	33	36	55[8]
Medulloblastoma	30	19	11	15	14	1	0	10.5	9
Ependymoma	22	09	13	3	12	7	0	14.5	19
Cavernoma	16	6	10	0	11	5	0	30.5	37[9]
Information unavailable	71	29	34	11	29	19	4		
	1232	588 (47.73%)	644 (52.27%)	60 (4.87%)	314 (25.49%)	746 (60.55%)	112 (9.09%)		

[a]Krishna Institute of Medical Sciences registry

[b]Data obtained from CIBTUS and references

medulloblastoma, ependymoma and cavernoma (Table 1). Among these types, astrocytomas were most common with 49.52% followed by meningioma of 21.67% and schwannoma of 17.53% (Fig. 1). Among astrocytomas diffuse astrocytoma (low grade) cases were most common with 37.87%. Overall, high grade astrocytoma cases (Anaplastic astrocytoma 15% and glioblastoma multiforme 33%) were reported with 48% and low grade astrocytoma (diffused astrocytoma 38% and pilocytic astrocytoma 8%) with 46% (Fig. 2).

In 6.07% cases accurate information about grade distribution was not available. Females were found to be more prone in all brain tumor type. Except in astrocytoma and medulloblastoma 63 and 58% male population was reported. Meningioma covers more than 70% female incidences followed by cavernoma 63%; ependymoma 59% and schwannoma 58% (Fig. 3a). Overall, high grade astrocytoma was recorded 58.1% of male and 41.9% of female cases (Fig. 3b).

Meningiomas are the second common diagnosed tumors in our registry. In total cases, 70.41% are females and 29.59% males. Schwannomas represents the third frequent brain tumors consisting of 58.8% females and 41.2% males. As per directory records, 4.87% cases are below 10 years old, 25.49% cases in range of 10–30 years, 60.55% in range of 31–60 years while 9.09% cases are above 60 years old. Most common treatment for high grade glioma tumors were surgical resection followed by post-operative chemo and radiation therapy.

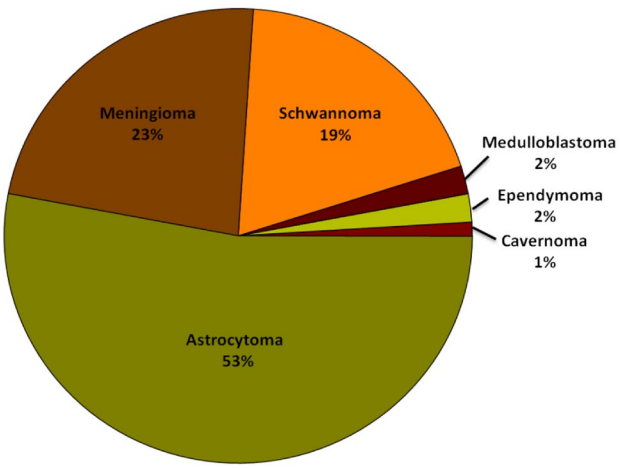

Fig. 1 Statistical distribution of brain tumors among different age groups in analyzed cohort

Discussion

Cancer registration system in developed countries reports classified information as annual incidence cases, relative survival rate, risk age of cancer development, relative death rate, etc. This information registry helps to predict the survival outcome of patients with provided treatment, risk age group, tumor location information which is in turn useful in generating effective therapeutic options.

National Cancer Institute (USA) has reported ethnic origin of population may contribute to alteration in cancer

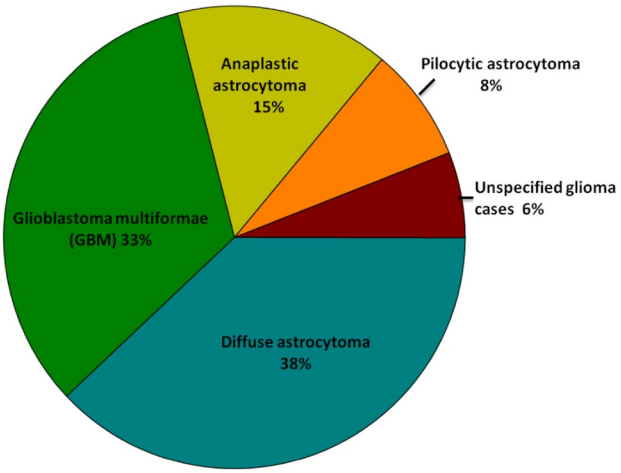

Fig. 2 Statistical distribution of astrocytoma subtypes among different age groups in analyzed cohort

associated mortality rates [2]. Genetic variation in particular ethnic population could be one of the factor for disparity. This could be the reason we cannot use foreign statistical information to reform therapeutic efficacy in Indian scenario.

In this study we have investigated the statistical distribution of brain tumor, median age and initial symptom and tried to confront actual scenario of Indian population as compare to CBTRUS and NCI annual reports.

Our data showed that in all brain tumor types, astrocytoma was most prevalent with 53 % followed by meningioma 23 %, schwannoma 19 %, medulloblastoma 2 %, ependymoma 2 % and cavernoma 1 % (Fig. 1). Overall, cancer was more prevalent among females with 52 % (Fig. 3a, b). In astrocytoma subtypes, diffuse astrocytoma was most common with 38 % followed by glioblastoma multiformae with 33 % (Fig. 2). Among meningioma subtypes, grade I (GI) was most frequent with 86.5 % followed by grade II (GII) with 10.11 % and grade III (GII) with 0.74 %. Increased rate of incidence of low grade meningioma was consistent with the existing literature [11]. The interesting fact we found is variation in median age in Indian population comparing to developed countries. The median age for pilocytic astrocytoma was 15.5 years in Indian population while data from CIBTUS reported the same as 23 years. Diffuse astrocytoma was found to affect considerably older population in India with median age of 54 year while it was 33 year in U.S. population. Anaplastic astrocytoma reported with median age of 45 in Indian population while it was 49 in U.S. population. Considerable variation was observed among median age of glioblastoma multiformae population. In India, it was 37.5 while 62 in U.S. population. Indian population was found to

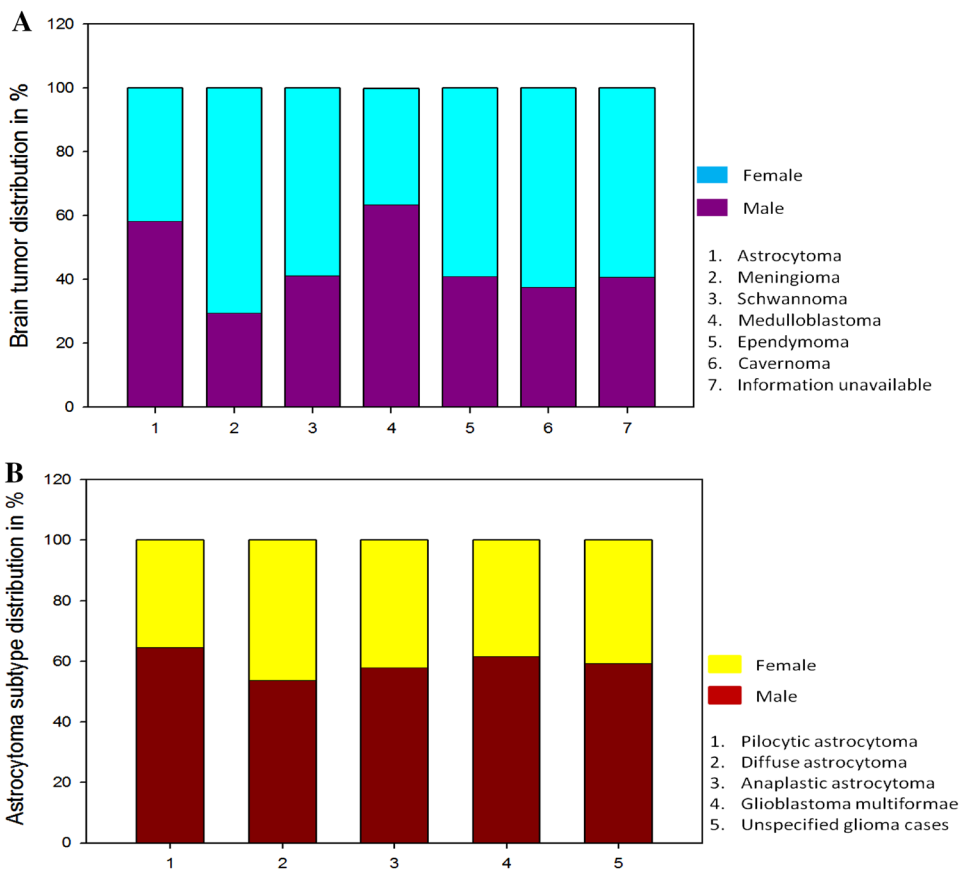

Fig. 3 Gender wise statistical distribution of brain tumors (**a**) and astrocytoma sub types (**b**)

Table 2 Most common symptoms associated with of astrocytoma subtypes

Sign or symptom	Astrocytoma type					
	Pilocytic astrocytoma (%)	Diffuse astrocytoma (%)	Anaplastic astrocytoma (%)	Glioblastoma multiformae (%)	Total distribution[a] (%)	Total distribution[b] (%)
Headache	6.8	6.83	7.63	10.84	49	56
Seizures	3.62	4.81	10.04	27.7	29	32
Vomiting	2	1.20	3.62	5.62	12.5	13
Memory loss	0.00	0.40	0.40	3.61	5	35
Hearing impairment	0.80	0.00	1.20	0.00	2	–
Vision loss	1.20	0.00	0.80	0.80	3	22

[a]Krishna Institute of Medical Sciences registry

[b]Data obtained from reference [10]

succumb to glioblastoma multiformae at much early age as compared to U.S. population. Median age for meningioma, schwannoma, medulloblastoma, ependymoma and cavernoma in Indian population was 43.5, 36, 10.5, 14.5 and 30.5 year respectively while in developed countries, it was 55, 55, 9, 19.3 and 37 years. Among the different age groups selected, people less than 10 years old were found to be least affected with 4.87 % while middle aged (30–60) population was most affected with 60.55 % (Table 1).

Developing countries have been shown to report less malignant brain tumors than in developed countries [12]. Accessibility to proper diagnostic and therapeutic practices, better healthcare measures and efficient registry system could be the possible reasons for differences in reporting of incidence rates.

Cytomegalovirus (CMV) infections were reported to be associated with occurrence of malignant glial tumors with vivid expression of its early and late gene products [13]. Further, peripheral blood of 80 % newly diagnosed glioblastoma patients were found with detectable CMV DNA while normal healthy subjects were devoid of infection [14]. However role of CMV infections and seropositivity in glioma biology remains to be substantiated owing to restraints of present diagnostic practices [15–17]. CMV infections and glioma incidences are associated with socioeconomic status. CMV infections in adulthood may be a risk factor for glioblastoma development while infection in early childhood could be protective [18]. In a developing country like India, the feasible association between CMV infections and glioblastoma incidence is yet to be investigated.

Among astrocytoma subtypes, headache was observed (Table 2) most prevalent with 49 % followed by seizures with 29 %. Vomiting, memory loss, hearing impairment and vision loss was observed with 12.5, 5, 2 and 3 %. We have compared this data with data from developed countries. Headache was reported with 56 % as compared to 46 % in Indian population. Seizures and vomiting was observed with 32 and 13 % in developed countries. Deviation was observed in vision loss and memory loss symptoms. In developed countries, vision loss and memory loss was observed with 22 and 35 % which was noted with only 3 and 5 % in our registry.

In conclusion, our data reported astrocytomas are highly reported neoplasms of central nervous system with high incidence in middle aged groups'. Among astrocytoma subtypes, diffuse astrocytoma was more prevalent followed by glioblastoma multiformae. Headache and seizures were observed more frequently in high grade glioma as compared to low grade glial tumors. Deviation was observed in median age of patients with developed countries. In meningiomas, females were reported with high incidence suggesting plausible role of female sex hormones in tumor progression.

Acknowledgments The authors thank department of pathology (Krishna Institute of Medical Sciences) for assistance with histopathological staining and patient registry, DBT for student fellowship, DST (No SB/EMEQ-257/2013) and UPE (UH/UGC/UPE-2 Interface studies/Research Projects/B1.4) for lab funding.

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Cellular and Molecular Neurobiology

Pantoprazole induces mitochondrial apoptosis and attenuates NF-κB signaling in glioma cells

--Manuscript Draft--

Manuscript Number:	CEMN-D-18-00163R1	
Full Title:	Pantoprazole induces mitochondrial apoptosis and attenuates NF-κB signaling in glioma cells	
Article Type:	Original Article	
Keywords:	Glioma; V-ATPases; pantoprazole; anticancer; apoptosis; NF-κB signaling.	
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Order of Authors Secondary Information:		
Funding Information:	University with potential for excellence, India (Grant no:UH/UGC/UPE-2 Interface studies/Research Projects/B1.4)	Prof. Phanithi Prakash Babu
	Department of Science and Technology, India (Grant no:SB/EMEQ-257/2013)	Prof. Phanithi Prakash Babu
Abstract:	Gastric H+/K+-ATPase or vacuolar-ATPases (V-ATPases) are critical for the cancer cells survival and growth in the ischemic microenvironment by extruding protons from the cell. The drugs which inhibit V-ATPases are known as proton pump inhibitors (PPIs). In the present study, we aimed to evaluate the anticancer efficacy of pantoprazole (PPZ) and its consequences on NF-κB signaling in glioma cells. We have used MTT and clonogenic assay to shows PPZ effect on glioma cell growth. Propidium iodide and rhodamine 123 staining were performed to demonstrate cell cycle arrest and mitochondrial depolarization. Tunel staining was used to evidence apoptosis after PPZ treatment. Immunoblotting and immunofluorescence microscopy was performed to depict protein levels and localization respectively. Luciferase assay was performed to confirm NF-κB suppression by PPZ. Our results revealed PPZ treatment inhibits cell viability or growth and induced cell death in a dose and time dependent manner. PPZ exposure arrest G0/G1 cyclic phase, increased tunel positivity, caspase-3 and PARP cleavage with altered pro and anti-apoptotic proteins. PPZ also induced ROS levels and depolarized mitochondria (Δψm) with increased cytosolic cytochrome c level. Further, PPZ suppressed TNF-α stimulated NF-κB signaling by repressing p65 nuclear translocation. NF-κB luciferase reporter assays revealed significant inhibition of NF-κB gene upon PPZ treatment. PPZ exposure also reduced the expression of NF-κB associated genes such as cyclin-D1, iNOS and COX-2 indicate NF-κB inhibition. Altogether, the present study disclosed that PPZ exerts mitochondrial apoptosis and attenuate NF-κB signaling suggesting PPZ can be an effective and safe anticancer drug for glioma.	

Response to Reviewers:	Dear Editor, We thank the reviewers for the constructive comments. The comments have been answered and incorporated in the manuscript. We believe that the quality of the manuscript is now further enhanced. Reviewer #1: In this study, authors reported for the first time that pantoprazole induced glioma cell death by inducing mitochondrial apoptosis and attenuating NF-κB signaling. This study is well designed and clearly written. However, minor revision is required to meet the criteria for publication. 1. The proton pump inhibitors are clinically used for the treatment of gastritis and gastric ulcers. Has it been shown that these inhibitors could cross the blood-brain-barrier? The efficacy of brain delivery is important to bring the clinical argument of using these drugs for glioma treatment, which need to be discussed. Yes, pantoprazole (PPZ) can cross blood brain barrier (BBB) (Sigaroudi et al. 2016); In fact, Breedveld et al 2005, study revealed PPZ co-administration can improved CNS delivery of imatinib, thereby suggesting that PPI may increase the efficacy of chemotherapy through a mechanism in addition to pH modulation (Breedveld et al. 2005). References Breedveld P, Pluim D, Cipriani G, Wielinga P, van Tellingen O, Schinkel AH, Schellens JH (2005) The effect of Bcrp1 (Abcg2) on the in vivo pharmacokinetics and brain penetration of imatinib mesylate (Gleevec): implications for the use of breast cancer resistance protein and P-glycoprotein inhibitors to enable the brain penetration of imatinib in patients. Cancer Res 65 (7):2577-2582. doi:10.1158/0008-5472.CAN-04-2416 Sigaroudi A, Stelzer C, Braun T, Frechen S, Huttner S, Schroter M, Kinzig M, Fuhr U, Holzgrabe U, Sorgel F (2016) Comparison of Pantoprazole Concentrations in Simultaneous Cerebrospinal Fluid and Serum Samples. Pharmacology 98 (1-2):70-72. doi:10.1159/000445720 2. In the introduction, authors mentioned that PPIs has little or no side effects up to 150 mg/d. Has any study reported that the brain concentration of PPIs at this dosage in human? It is compared to the concentration used in the current study? We regret the written dose (150 mg/d), in context of the safety concern, we have mentioned that the short or long term PPZ administration even at the higher dose up to 120mg/day showed good safety profile (Martin de Argila 2005; Mathews et al. 2010). But to the best of our knowledge, there is no report which speaks about PPZ concentration in the brain at this dosage in the human body. In the current study for the first time, we are reporting the anticancer efficacy of PPZ in glioma in-vitro. Initially, we used nine different concentration of PPZ to calculate the IC50 value of PPZ on glioma cells as mentioned in the manuscript in the support of earlier report (Yeo et al. 2006). References Martin de Argila C (2005) Safety of potent gastric acid inhibition. Drugs 65 Suppl 1:97-104 Mathews S, Reid A, Tian C, Cai Q (2010) An update on the use of pantoprazole as a treatment for gastroesophageal reflux disease. Clin Exp Gastroenterol 3:11-16 Yeo M, Kim DK, Han SU, Lee JE, Kim YB, Cho YK, Kim JH, Cho SW, Hahm KB (2006) Novel action of gastric proton pump inhibitor on suppression of Helicobacter pylori induced angiogenesis. Gut 55 (1):26-33. doi:10.1136/gut.2005.067454 3. In Figure 5a, the protein level of pIKB-a and Ikka is increased by 200 uM PPZ compared to control, which was decreased with high concentration. I am wondering if authors have a potential explanation for these results. In the present study we showed PPZ treatment supresses NF-kB signaling even upon

TNFα stimulation by repressing p65 nuclear translocation, as suggested by reviewers we repeated and rectified the western blots of pIKB-a and Ikka proteins in Figure 5a, we observed that at 200 uM of PPZ concentration there is very low or no inhibition of pIKB-a and Ikka proteins as compared to the control but with increase in PPZ dose significant inhibition was observed.

4. The resolution of all the figures is poor, which need to be improved.

We have improved the resolution of all the figures.

Research article

Title: Pantoprazole induces mitochondrial apoptosis and attenuates NF-κB signaling in glioma cells

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Abstract

Gastric H^+/K^+-ATPase or vacuolar-ATPases (V-ATPases) are critical for the cancer cells survival and growth in the ischemic microenvironment by extruding protons from the cell. The drugs which inhibit V-ATPases are known as proton pump inhibitors (PPIs). In the present study, we aimed to evaluate the anticancer efficacy of pantoprazole (PPZ) and its consequences on NF-κB signaling in glioma cells. We have used MTT and clonogenic assay to shows PPZ effect on glioma cell growth. Propidium iodide and rhodamine 123 staining were performed to demonstrate cell cycle arrest and mitochondrial depolarization. Tunel staining was used to evidence apoptosis after PPZ treatment. Immunoblotting and immunofluorescence microscopy was performed to depict protein levels and localization respectively. Luciferase assay was performed to confirm NF-κB suppression by PPZ. Our results revealed PPZ treatment inhibits cell viability or growth and induced cell death in a dose and time dependent manner. PPZ exposure arrest G0/G1 cyclic phase, increased tunel positivity, caspase-3 and PARP cleavage with altered pro and anti-apoptotic proteins. PPZ also induced ROS levels and depolarized mitochondria ($\Delta\psi m$) with increased cytosolic cytochrome *c* level. Further, PPZ suppressed TNF-α stimulated NF-κB signaling by repressing p65 nuclear translocation. NF-κB luciferase reporter assays revealed significant inhibition of NF-κB gene upon PPZ treatment. PPZ exposure also reduced the expression of NF-κB associated genes such as cyclin-D1, iNOS and COX-2 indicate NF-κB inhibition. Altogether, the present study disclosed that PPZ exerts mitochondrial apoptosis and attenuate NF-κB signaling suggesting PPZ can be an effective and safe anticancer drug for glioma.

Keywords: Glioma; V-ATPases; pantoprazole; anticancer; apoptosis; NF-κB signaling.

Manuscript Details

Manuscript number CMLS-D-18-01038

Article type Original Article

Title: Portrayal of EMT like process in human glioma and its impediment by rabeprazole to combat glioma growth and temozolomide resistance

Deepak Babu[1], Anwita Mudiraj[1], Chintal Ramulu[1], Manas Panigrahi[2] and Phanithi Prakash Babu[1*] [1]Neuro Science Laboratory, Department of Biotechnology and Bioinformatics, School of Life Sciences, University of Hyderabad, Hyderabad, 500 046, Telangana State, India

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Running Title: Clinical and therapeutic significance of EMT

Translational Relevance

PPIs anticancer and chemosensitization efficacy has been endorsed by several studies but its appropriate administration to the patients remains lacking. Here, we unveil the clinical significance of EMT like process in human glioma and its therapeutic relevance to rabeprazole treatment along with rabeprazole anticancer and chemosensitization efficacy; advocating rabeprazole can be a potential anticancer drug.

Abstract

Purpose

The acidic microenvironment of the tumor accelerates epithelial to mesenchymal transition (EMT) and NF-κB activation aggravating glioma malignancy and chemoresistance. Here, we investigated the clinical significance of EMT like process and its therapeutic potential using rabeprazole in glioma.

Experimental Design

To characterize the EMT like process in glioma we investigated the expression profile of EMT associated proteins in clinical biopsies and correlated with various clinicopathological features. We also demonstrate the therapeutic relevance of EMT along with the anticancer and temozolomide chemosensitization by rabeprazole using multiple cell-based assays. Further, we validate *in-vitro* outcomes using C6 stereotaxic rat glioma model.

Results

The expression analysis of EMT associated proteins revealed the non-canonical expression of E-cadherin or N-cadherin and upregulation of GFAP, vimentin, and β-catenin in human glioma that may attribute to glioma malignancy and poor prognosis. Rabeprazole treatment attenuated glioma cell growth, cell migration and induced apoptosis *in-vitro* and *in-vivo*. Rabeprazole exposure suppressed EMT by repressing NF-κB signaling and/or inhibiting Akt/GSK3β phosphorylation. Further, the *in-vivo* study corroborates *in-vitro* results with improved animal survival in the confluence of EMT inhibition. We also showed temozolomide sensitizing effect of rabeprazole on temozolomide resistant cell line.

Conclusions

The two major finding of our study: (i) we evidenced the clinical association of EMT like process in glioma malignancy and poor prognosis and (ii) its therapeutic relevance to rabeprazole treatment along with rabeprazole anticancer and chemosensitization efficacy; warrants rabeprazole repurposing for therapeutic interventions.

Keywords: Glioblastoma multiforme; Epithelial to mesenchymal transition; Proton pumps, Rabeprazole; Anticancer.

Manuscript Details

Manuscript number ID 18-533

Article type Research Article

Title: Distinct expression and role of breast cancer metastasis suppressor 1 (BRMS1) in *mut*P53 glioblastoma

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Running Title: BRMS1 significance in *mut*P53 GBM

Abbreviations: GBM, Glioblastoma multiforme; BRMS1, Breast cancer metastasis suppressor 1; *wt*/*mut*P53, wild/mutant P53; UPA, Urokinase plasminogen activator; ING4, Inhibitor of growth family-4; siRNA, Small interfering RNA.

Abstract

Glioblastoma multiforme (GBM) is a noted malignancy of central nervous system with a poor prognosis among all malignant forms. Breast cancer metastasis suppressor 1 (BRMS1) suppresses metastasis in multiple tumors, but its clinical and molecular importance in *mut*P53 GBM is not known. In the present study, we have investigated the clinical significance of BRMS1 and its functional relevance in *mut*P53 GBM. BRMS1 expression was evaluated in human astrocytoma tissue (n=74) by RT-qPCR, western blotting and immunohistochemistry. BRMS1 expression was analyzed with various clinicopathological parameters and correlated with *wt/mut*P53 and *BRMS1* downstream targets *uPA* and *ING4* (metastasis biomarker). The *BRMS1* siRNA transfection study was performed to elucidate the role of BRMS1 in GBM. Our findings revealed overexpression of BRMS1 in malignant astrocytoma associated with pathological grading and patient's poor survivability. We observed the positive correlation between BRMS1 and *mut*P53 in GBM tissues which may extort GBM malignancy and poor prognosis. The *in-vitro* siRNA study revealed reduced cell growth and survivability with increased apoptotic proteins in the *mut*P53 cell line. Also, *BRMS1* knockdown suppressed EGFR-AKT signaling and NF-κB proteins in *mut*P53 cells. Further, the transcript profiling disclosed a positive and negative correlation of *uPA* and *ING4* with *BRMS1* respectively. Altogether, we report an upregulation of BRMS1 in *mut*P53 glioblastoma worsens GBM malignancy and patient's survivability. The *BRMS1* knockdown restricted cell growth and sensitized apoptosis by modulating EGFR-AKT signaling in *mut*P53 GBM cells, suggesting BRMS1 can be a prognostic and therapeutic target for *mut*P53 GBM.

Key words: Glioblastoma multiforme; BRMS1; *mut*P53, therapeutic target.

Manuscript Details

Manuscript number	APSB_2018_599
Title	Therapeutic implication of plumbagin in CTTN-EGFR modulated astrocytoma malignancy
Short title	CTTN-EGFR after plumbagin
Article type	Full Length Article
Authors detail	Deepak Babu[1], Chintal Ramulu[1], Manas Panigrahi[2] and Phanithi Prakash Babu[1*]

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Abstract

Glioblastoma multiforme (GBM) is the deadliest malignancy of primary brain tumors with least (5.5 %) post diagnostic cancer survival. Cortactin (CTTN) is reported to be upregulated in various cancers, including glioma. Plumbagin (PL), a plant-derived product has also been evidenced as an anticancer drug by inducing apoptotic cell death. In the present study, we report the clinical importance of CTTN-EGFR in GBM and its therapeutic implication by PL. We demonstrated the overexpression of CTTN in GBM as compared to the low grades and control tissues. The clinicopathological analysis revealed increased CTTN expression is significantly correlated with grade progression and patients' poor survival. We observed the positive association between CTTN and EGFR in astrocytoma grades and their positivity inflicts dismal patients' survival. Further, PL exposure even at low concentration (2μM) significantly inhibits CTTN and EGFR in GBM cell lines. The *in-vitro* PL treatment induced apoptotic cell death and reduced cell survival and/or cell migration in GBM cells by repressing CTTN modulated EGFR-AKT signaling. Overall, the present study evidenced the clinical association of CTTN and EGFR in astrocytoma malignancy and patients' poor survival together with its therapeutic relevancies to PL by suppressing EGFR-AKT signaling.

Graphical abstract

CTTN etiology in carcinogenesis has been evidenced by several studies but its therapeutic importance remains elusive. Here, we disclose the clinical significance of CTTN in human astrocytoma and its therapeutic relevance to plumbagin by CTTN-EGFR repression.

Key words: Glioblastoma; Cortactin; EGFR; Plumbagin; Therapeutic target

Abbreviations: Glioblastoma multiforme (GBM), Cortactin (CTTN), Plumbagin (PL), Epidermal growth factor receptor (EGFR), Protein kinase B (AKT).

Clinical and therapeutic significance of epithelial to mesenchymal transition (EMT) like process in glioma

by Deepak Babu

Clinical and therapeutic significance of epithelial to mesenchymal transition (EMT) like process in glioma

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