Retrospective clinical analysis and pathological significance of biomarkers for astrocytoma progression

A Thesis Submitted to the University of Hyderabad, for the award of Doctor of Philosophy in Department of Biotechnology & Bioinformatics

By

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DECLARATION

I, Deshpande Ravindra Pramod hereby declare that this thesis entitled **"Retrospective clinical analysis and pathological significance of biomarkers for astrocytoma progression"** submitted by me under the supervision of Prof. P. Prakash Babu is an original and independent research work. I also declare that it has not been submitted previously in part or in full to this University or any other University or Institution for the award of any degree or diploma. The particulars given in this thesis are true to the best of knowledge and belief. I hereby agree that my thesis can be deposited in Shodaganga/INFLIBNET. A report on plagiarism statistics from the University Library is enclosed.

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Parts of this thesis have been-

A. published as following research papers:

1. **Deshpande RP**, Chandra Sekhar YB, Panigrahi M, Babu PP. Region specific Dok2 overexpression associates with poor prognosis in human astrocytoma.

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2. **Deshpande RP**, Chandra Sekhar YB, Panigrahi M, Babu PP. SIRP Alpha protein downregulates in human astrocytoma: presumptive involvement of Hsa-miR-520d-5p and Hsa-miR-520d-3p. **Molecular Neurobiology**. 2016. pp 1–8. doi:10.1007/s12035-016-0302-8. ISSN: 1559-1182.

3**. Deshpande RP**, Babu D, Panigrahi M, Chandra Sekhar YB, Prakash Babu P. Brain tumors incidences and a retrospective clinical analysis from a tertiary hospital in India.

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List of abbreviations:

Glioblastoma multiformae	: GBM
Glioblastoma with oligodendroglial component	: GBMO
Central brain tumor registry of United States	: CBTRUS
Anaplastic astrocytoma	: AS
Magnetic resonance imaging	: MRI
Mitogen activated protein kinase	: MAPK
Docking protein 2	:Dok2
World Health Organization	: WHO
Phosphatase and tensin homolog	: PTEN
Vascular endothelial growth factor	: VEGF
orphan receptor 2	: Ox-2R
Signal regulatory protein alpha	: SIRP Alpha
Immunohistochemistry	: IHC
Src homology region 2 domain-containing phosphatase-1	: SHP 1
non-catalytic region of tyrosine kinase	: Nck
Mammalian target of rapamycin	: mTOR
Eukaryotic Initiation Factor 2	: elf2
Nitidine chloride	: NC
focal adhesion kinase	: FAK
Glycogen synthase kinase 3	: Gsk3
Millimolar	: mM
Micromolar	: μM

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Chapter I

Introduction

1.1 Introduction:

Astrocytoma's are the most common malignancies of the brain. World Health Organization (WHO) has classified astrocytoma in four grades (Grade I- pilocytic astrocytoma, Grade II- diffuse astrocytoma, Grade III- anaplastic astrocytoma and Grade IV- glioblastoma multiforme) on account of cellularity, nuclear polymorphism, mitotic index, microvascular proliferation and extent of necrosis [1]. However recent scheme of classification based on the co-expression modules around most mutated gene such as receptor tyrosine kinases are proposed to accurately assign prognosis in astrocytoma cases[2]. Grade IV astrocytoma also referred as glioblastoma multiformae (GBM), are the most aggressive primary tumours with worst prognosis and account for nearly 60% of malignant gliomas [3-5]. The overall prognosis for malignant glial tumours have not changed significantly since 1980 despite of advancements in course of diagnosis and mode of treatment [6].

1.1.1 GI astrocytoma (Pilocytic astrocytoma):

The term 'pilocytic' refers to hair like bipolar process. Pilocytic astrocytoma (PA) is also referred to as optic glioma, cerebellar astrocytoma, and infundibuloma on account of its occurrence and unique predilection for young patients and certain anatomic sites, including the third ventricular/hypothalamic region and cerebellum. PA are most common among the children, constitutes 5.1% of astrocytomas and are more common in males[7]. PA are reported to have good prognosis with 10 year survival rate of 90%. Primarily, PA are treated by surgery. It may be followed by radiotherapy when complete resection is not possible. Chemotherapy is followed in event of tumor progression especially when surgery is not possible.

1.1.2 GII astrocytoma (Diffuse astrocytoma):

Diffuse astrocytoma (DA) are noted to be slow growing in their nature. On account of their diffuse infiltrative growth, DA are reported to have high recurrence rate and have potential to progress into high grade forms as anaplastic astrocytoma and secondary glioblastoma. DA are characterized by moderate cellularity and nuclear atypia, low mitotic activity, absence of necrosis, and microvascular proliferation and are noted with median survival rate of 5-8 years. DA are treated by surgical resection (biopsy, gross total resection, partial resection) followed by chemo and radiotherapy [8].

1.1.3 GIII astrocytoma (Anaplastic astrocytoma):

Anaplastic astrocytoma (AS) represents the infiltrating tumours of the diffuse astrocytic and oligodendroglial group. 2016 WHO classification has categorized AS into subgroups on account of IDH mutations (IDH mutant, IDH wild type) [9]. Histologically they are more infiltrative than diffuse astrocytomas with visible nuclear atypia and pleomorphism. AS are reported with average prognosis of 2-3 years.

1.1.4 Grade IV Astrocytoma (Glioblastoma multiformae):

Glioblastoma multiformae (GBM) represents the most aggressive from of astrocytic tumours with worst prognosis (9-12 months). GBM are reported to arise by two pathways: primary or *de novo* and secondary GBM. Primary GBM is known to arise without previous history of low grade tumours and found mostly in elder population while secondary GBM progresses from previous low grade forms (Fig. 1). Histologically, GBM is multiforme microscopically, with the regions of necrosis, pleomorphic nuclei and microvascular proliferation. On account of its diffuse nature, GBM is not curable by surgery and subject to recurrence. Standard care of treatment for GMB remains same since long time. It comprises of surgical resection followed by chemo and radiotherapy. Chemotherapy is usually intended to damage DNA or inhibit replication.

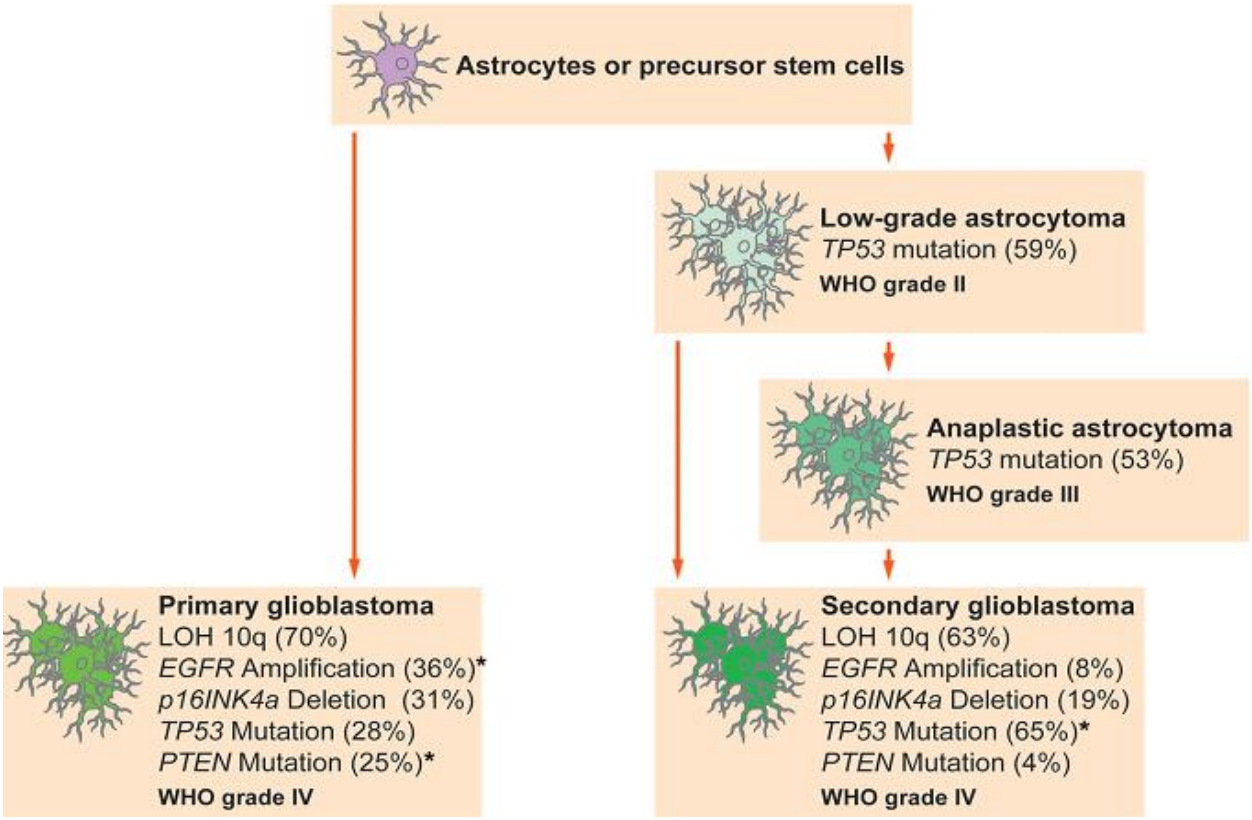

Fig.1: Pathways leading to glioblastoma progression. Primary glioblastoma is known to arise without any previous history of low grade tumors while secondary glioblastoma occur with previous onset of low grade tumors. LOH 10q is most frequently observed with primary glioblastomas while p53 mutations are common in secondary glioblastomas [10].

Table 1: WHO grading summary:

WHO grade	% survival (5 Years)	Treatment practiced	Histology's	Median survival
Grade I (**Pilocytic astrocytoma**)	91%	Biopsy. Chemotherapy given to young children instead of radiotherapy.	Low to moderate cellular differentiation, few mitosis or endothelial proliferation.	2-10 years
Grade II **(Diffuse astrocytoma)**	47%	Depends on size and location of tumours. Partial resections treated with chemo or radiotherapy.	Low to moderate cellular differentiation with cellular atypia.	2-10 years
Grade III (**Anaplastic astrocytoma**)	29%	Surgery depends on location and size of tumours. It is followed by radio or chemotherapy	Increased cellularity as compared to GII. Nuclear atypia. Mitoses present. No necrosis and vascular proliferation	2-3 years
Grade IV **(Glioblastoma multiformae**)	3%	Near total or subtotal resection followed by radiotherapy or combined radio and chemo therapy.	High cellularity, vesicular proliferation. Necrosis is present.	9-12 months

1.2 Causes and risk factors:

Although many potential risk factors (environmental as ionizing radiation and genetic risk factors) are being studied, there are no clear evidences that clearly points to the cause of astrocytic tumours. X ray exposure to head is estimated to cause less than 5% brain tumors. The use of immunosuppressive drugs and immune system disorder (such as AIDS) are also predicted but less extensively studied.

1.3 Statistics:

Brain tumors are consequences of abnormal cell growth in brain, contributing total 256,213 new cases reported globally in 2012. The estimated incidence rate is 68,470 in USA in the year 2015

Gliomas accounts for 27% of all the tumors and 80% of malignant tumors (Fig. 2). Astrocytomas including glioblastomas accounts for 75% of all the gliomas. [13]. Overall in all races, males are reported to be more prone to brain tumors than females with 7.7 males and 5.4 females per 100,000 persons [14].

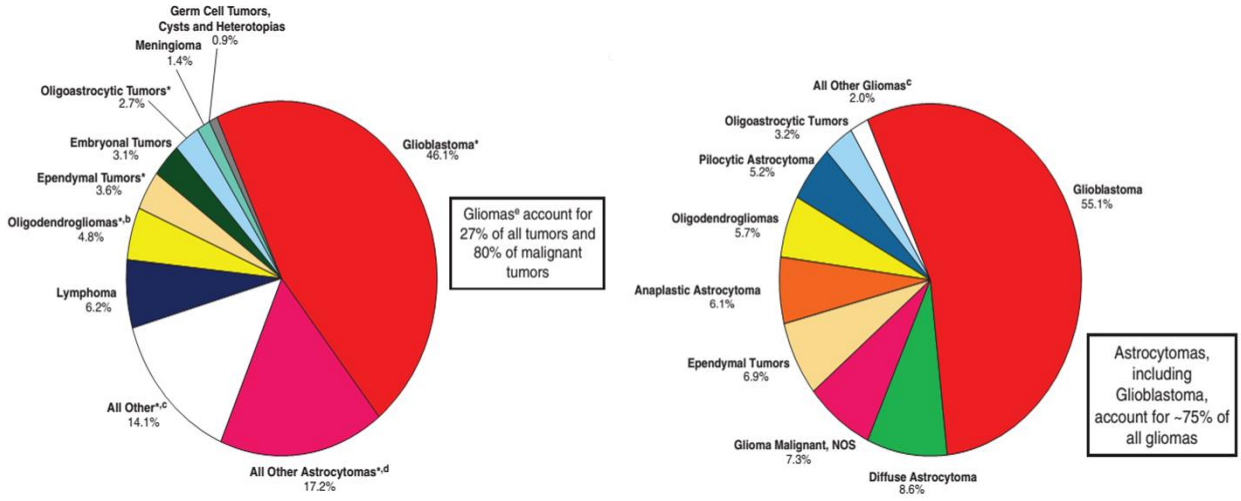

Fig.2: CBTRUS statistical report: Primary brain and central nervous system tumors diagnosed in the United States in 2008-2012[11]

1.4 Symptoms:

Symptoms of astrocytoma depends on the location of tumor and in turn the region affected. For example, most pilocytic astrocytoma are located in cerebellum, so most commonly associated symptoms are related to balance and coordination difficulties. However, the most commonly observed symptoms among four astrocytoma grades are persistent headache, seizures, blurred vision, and changes in mood.

1.5 Diagnosis:

Magnetic resonance imaging (MRI) is the most commonly operated method for astrocytoma detection. Computed tomography (CT) scans are also used however, the MRI imaging is more

sensitive and reliable than CT scanning. On initial diagnosis by MRI, surgery is performed. This scan measures the chemical and mineral level in tumor. These measurements suggest for malignant or benign nature of tumor. The surgically resected tissue is further processed for accurate histopathological diagnosis. Tumors are graded on account of several parameters as vascularity, mitotic index, presence of necrosis, invasive potential in terms of border distinctness and its similarity with normal cells [12].

1.6 Treatment:

The treatment practiced depends on the type of tumor, its location, age and health of patients. However, the most commonly followed practices are surgery radiotherapy and/or chemotherapy. Recently, molecular targeted therapies are also followed (Fig. 3).

1.6.1 Radiation therapy:

Radiation therapy is followed in cases where complete surgical resection is not possible. It can also be used after surgical resection to kill the residual tumor cells. Radiotherapy is generally not prescribed for young patients where they are prone to long term developmental changes.

1.6.2 Surgery:

A surgery can be a biopsy, partial resection, subtotal resection and gross total resection. The extent of surgical resection depends on location, size of tumor, health and age of patients. Recent advances in surgical procedures are also evident such as microsurgery, where high power microscopes are used which enables surgeons to remove aggressive tumor tissue while avoiding damage to critical structures and ultrasonic aspirations where tumors are fragmented by ultrasonic waves and the fragments are removed by suction.

1.6.3 Chemotherapy:

In chemotherapy, drugs are used to treat the tumor. Most commonly followed drug at present is temozolomide. It is an alkylating agent and induces abnormalities in DNA replication pathway of tumor cells. In addition to temozolomide, cytotoxic therapies as procarbazine-CCNU-vincristine (PCV) is also followed. Molecular targeted therapies are less frequent. Bevacizumab is most noted drug which is a selective inhibitor of vascular endothelial growth factor A. It is reported to slow the tumor growth but however do not have prognostic benefit [13] [31].

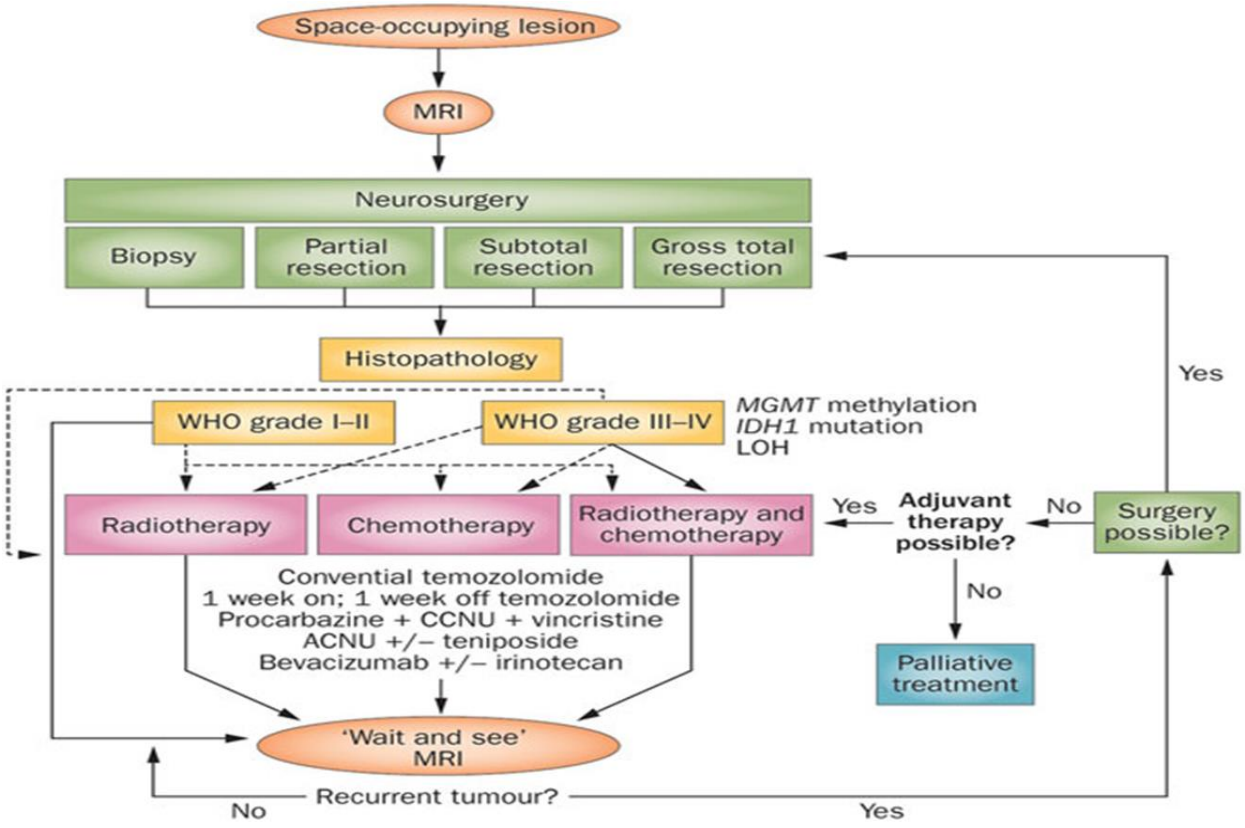

Fig.3: Diagnosis and treatment practice for astrocytoma. On initial detection by MRI imaging, surgical resection is followed. The extent of resection depends on size, location of tumor and health of patients. Further, chemo and/or radiotherapy is given. The most commonly used drug at present is temozolomide which alkylates the DNA and hampers the repair process [12].

1.7 Scope of present studies:

Despite present clinical advances, median age of patients' diagnosed with glioblastoma is 1-2 years [7]. This highlights the immediate need to understand the biology of glial tumours for possible therapeutic intervention. As described by WHO, glioblastoma with oligodendroglial component (GBMO) have been described as high grade astrocytic neoplasms with discrete oligodendroglial differentiation areas displaying necrosis [1]. Certain studies have claimed that glioblastoma with oligodendroglial component display better prognostic pattern, yet this hypothesis remains controversial.

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 tumours and observed symptoms. These data bases can help to study cancer profile in large cohort [14] 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 [11]. In 2008, a study from India reported by Jalali R. and Datta D. conclude that the rates of benign tumours are low in comparisons to malignant tumours in Indian population. This study also suggest the lower median age of patients in Indian population as compared to western countries [15]. The statistical studies in developing countries like India are underscored owing to limited access to the biological data and inefficient registry system.

Immunomodulatory receptors as CD200R1 and SIRP Alpha forms the immunological synapse between myeloid membrane and their ligands on other cells [16]. We have shown that SIRP Alpha is down regulated in human astrocytoma with presumptive involvement of miR-520d-5p and miR-520d-3p[17]. CD200R, previously known as OX2R is a transmembrane glycoprotein and widely expressed on myeloid cells [18, 19]. CD200R1 is reported to exert anti-inflammatory effect in

engagement with its ligand, CD200 [20] leading to exert inhibitory effect on mitogen activated protein kinase (MAPK) signaling [21].

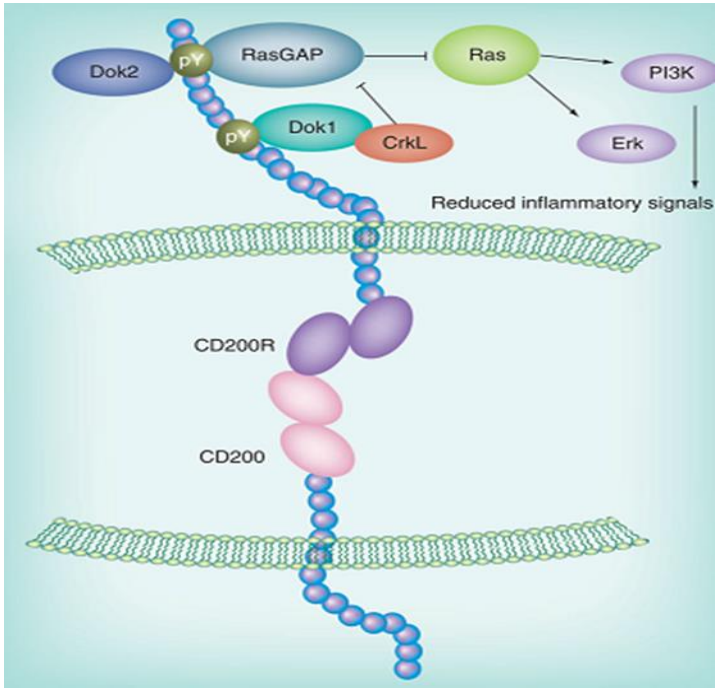

Fig.4: Presumptive mechanisms of CD200R1 signaling in neurons and microglia cells in brain. Primarily, upon binding between neuronal CD200 and microglial CD200R, downstream signaling involves adapter protein Dok2 and RasGAP and resulting in Ras inhibition. This, in turn is reported to lead to reduced inflammatory signaling [12].

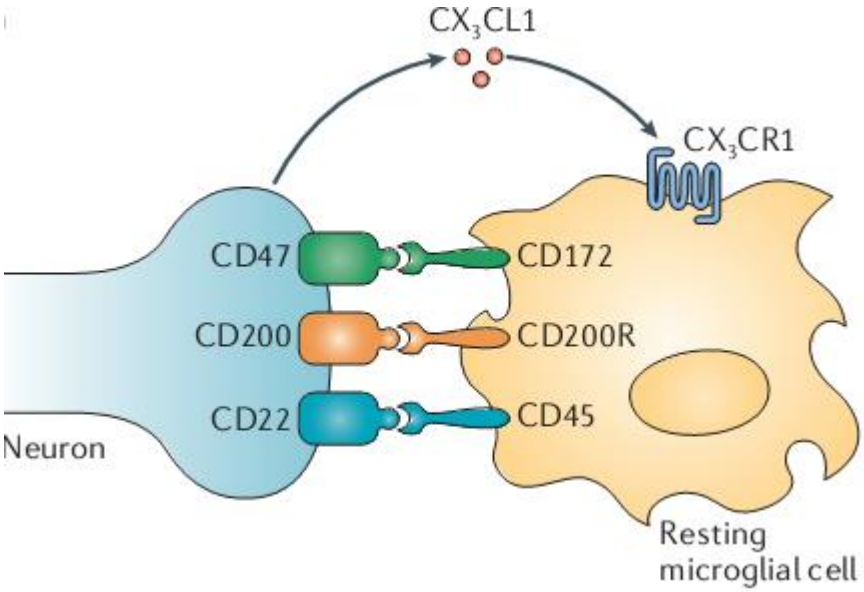

Fig. 5: Biology of CD200R and SIRP Alpha (CD172) interactions across neurons and microglial cells. CD200R and SIRP Alpha are the transmembrane proteins and expressed on immunological cells. These proteins are helpful in maintaining the steady cell state in microglial cells [22].

CD200R functions through a unique inhibitory pathway with Dok2 as one of the downstream component. This culminates in binding and activation of RasGAP [23]. This involves the progression of inhibitory signaling (Fig. 4, 5). Dok2 have been reported to play a crucial role in mediating the inflammatory response in response to CD200R in microglial cells [24]. Dok2 have been independently reported to be an important modulator in malignancies as gastric [25], colorectal cancer [26] Recently, Dok1 and Dok2 protein have been found to be associated with regulation of cell cycle in hematopoietic stem and progenitor cells [27].

Nck1 have been reported as a direct target of Dok2 and have a role on cellular migration and a downstream target of many growth factor receptors [28].

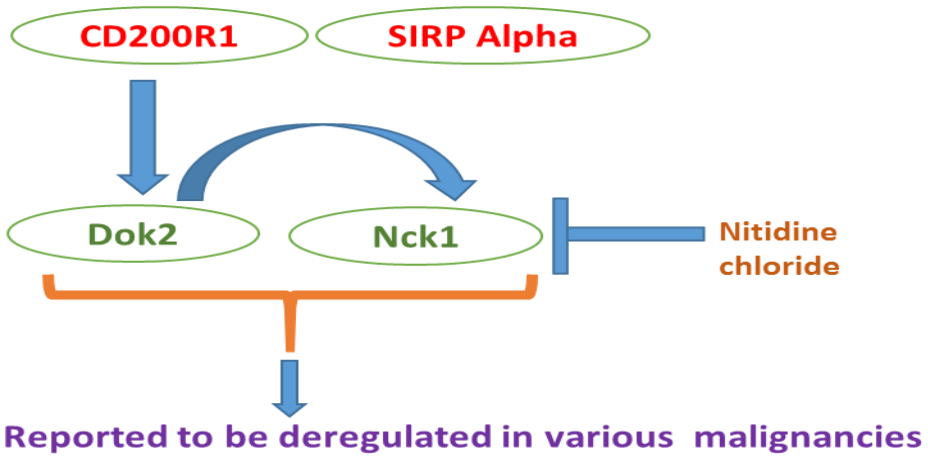

Fig. 6: Presumptive interaction of CD200R1 and its downstream targets Dok2 and Nck1. These two proteins have been reported to be deregulated in various malignancies as noted in text. We have looked for expression profile and significance of immunomodulatory receptors (CD200R1 and SIRP Alpha) in surgically resected tissue sample and in addition to expression profile, prognostically evaluated the role of Dok2 and Nck1 in astrocytoma tissue samples. Further, we have screened the nitidine chloride induced cell death in glioblastoma cell lines (U87 and C6).

Taken together, we have explored the expression profile of CD200R1 and SIRP Alpha in control brain and surgically resected low and high grade astrocytoma tissue samples. In addition to expression profile and significance, we have prognostically evaluated the importance of Dok2 and Nck1 protein in selected set of tissues and further evaluated the nitidine chloride mediated cell arrest in glioblastoma cell lines (Fig. 6).

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Chapter II

Retrospective clinical analysis of brain tumors- a tertiary experience.

a. Clinico-pathological assessment of astrocytoma

b. Prognostic significance of anatomic origin and glioblastoma with oligodendroglial component (GBMO) of astrocytoma patients'

a. Clinico-pathological assessment of astrocytoma

2 .1 Clinico-pathological assessment of astrocytoma:

2.1.1 Introduction:

2.1.2 Statistical distribution of astrocytoma: Worldwide scenario:

Brain tumors are consequences of abnormal cell proliferation in brain. It is reported to contribute to total 256,213 new cases reported globally in 2012. In USA, the estimated incidence rate is 68,470 in the year 2015 [1]. In all races, males are reported to be more prone to brain tumors as compared with female population. It is estimated to be 7.7 males and 5.4 females per 100,000 persons [2]. World Health Organization (WHO) has classified brain tumors into groups based on cellular origin and histopathological features [3]. Gliomas appear to be the most common primary brain tumors among all groups [1, 3]. In spite of aggressive chemo and radio therapy, the median survival age for progressive gliomas is still around 12-15 months [4].

Cancer databases with appropriate details are monitored in developed countries. The categorized study on account of information from the database provides population based information as tumor location, median age of detection; most commonly followed therapies, and observed symptoms. This database is helpful to study the statistical profile of cancers in large population [5].

2.1.3 Astrocytoma statistics in India:

Cancer registry in India is mostly maintained at tertiary levels in individual hospitals. National cancer registry program was undertaken by the Indian Council of Medical Research (ICMR) for epidemiological studies and registration of cancer cases [6]. A study from India in 2008, reported the rates of benign tumor are low in comparisons to malignant tumors. It also suggested the lower median age of patients in Indian population as compared to western countries [7]. However major limitation of such studies seems to be limited sample size and homogeneity of population.

2.2 Methods and Scope of present studies:

In the present study, we have analyzed 1232 patients and attempted to record clinico-pathological information from the hospital. It includes genders, median age of diagnosis of patients and cell specific types of brain tumor in studied population. In addition, we analyzed initial symptoms associated with the astrocytoma subtypes. This type of information may be useful in studying of demographic distribution of brain tumors in both sexes of Indian population.

A directory of patients was maintained at hospital with clinico-pathological parameters. In directory, each patient was assigned with a unique number. Clinical updates as age/sex were maintained in directory with information of symptoms, as date of surgery, date of radiation and chemotherapy and pathological reports. Grades of brain tumors were confirmed upon histological diagnosis from the pathology department of same hospital. The data was categorized in different tumor types with selected age group and gender ratios.

2.3 Results:

In present study, we have analyzed total 1232 cases and followed up for the period of 6 years (January 2009 to December 2014). Tumors were categorized in different types upon histological diagnosis such as astrocytoma (further subcategorized to four grades as per WHO classification), medulloblastoma, meningioma, schwannoma, ependymomas and cavernoma (Table 2). Among these cancer types, astrocytomas were most common with 49.52% followed by meningioma of 21.67% and schwannoma of 17.53% (Fig 7). In astrocytomas, diffuse astrocytoma cases were most common with 37.87%. Overall, high grade astrocytomas were reported with 47.7% while low grade astrocytomas with 46.2%. In 6.07% cases proper information about grade distribution was not available. Overall, females were found to be more prone in all brain tumors. Astrocytoma and medulloblastoma was found with 63% and 58% male population respectively. Female incidence rate was observed to be dominated in meningiomas with more than 70% followed by cavernoma with 63%, ependymomas with 59% and schwannoma with 58% (Fig.9a). Overall, high grade astrocytomas were recorded with 58.1% of male and 41.9% of female cases (Fig.9b).

Meningiomas were observed to be the second most common diagnosed tumors in our registry. In total meningioma cases, 70.41% were females and 29.59% were males. Schwannomas were noted to be the third frequent brain tumors with 58.8% females and 41.2% male population. As per the patient directory records, 4.87% cases were below 10 years old, 25.49% cases were in the range of 10-30 years, 60.55% were 31-60 years while 9.09% cases were found above 60 years. Most common treatment for high grade astrocytoma tumors were surgical resection followed by post-operative chemo and/or radiation therapy. Surgery and type of treatment was function of few parameters as location and size of tumor, health and age of patients.

Tumor type	Total cases	Male	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%)		

Table 2: statistics of distribution of central nervous system tumors and comparison with data from developed countries.

[a]Krishna Institute of Medical Sciences registry

[b]Data obtained from CIBTRUS and references

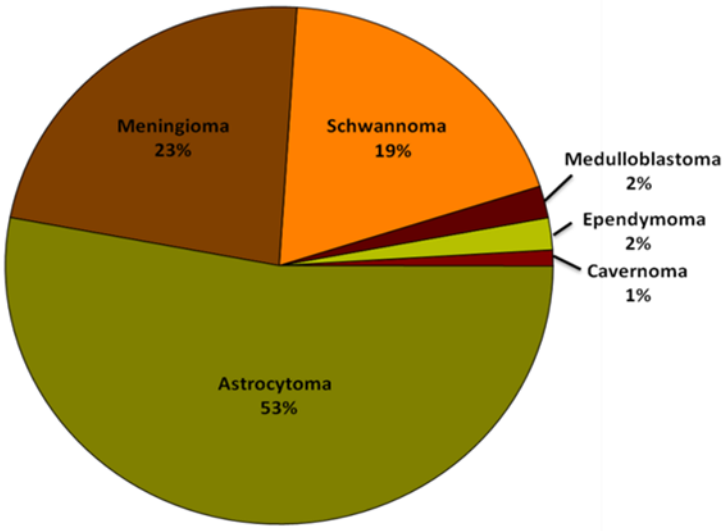

Fig. 7: Statistical distribution of brain tumors in analyzed group**.**

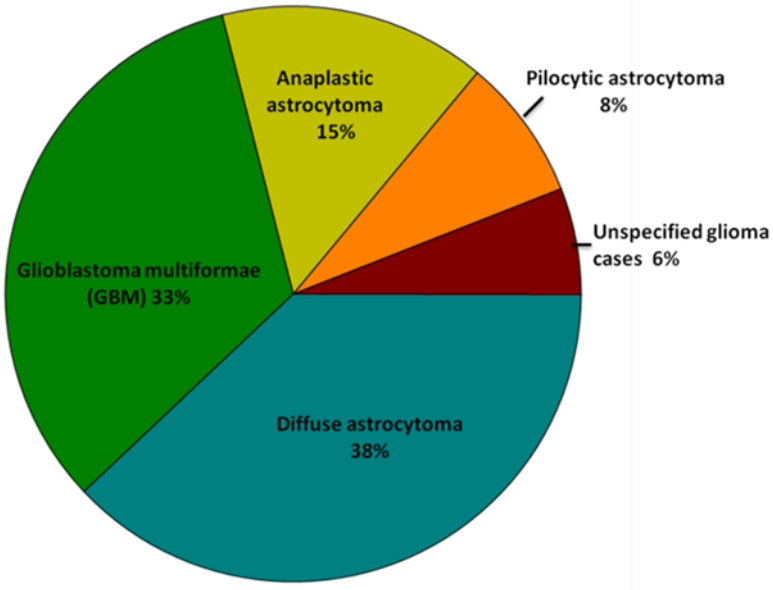

Fig 8: Statistical distribution of astrocytoma subtypes among in analyzed group.

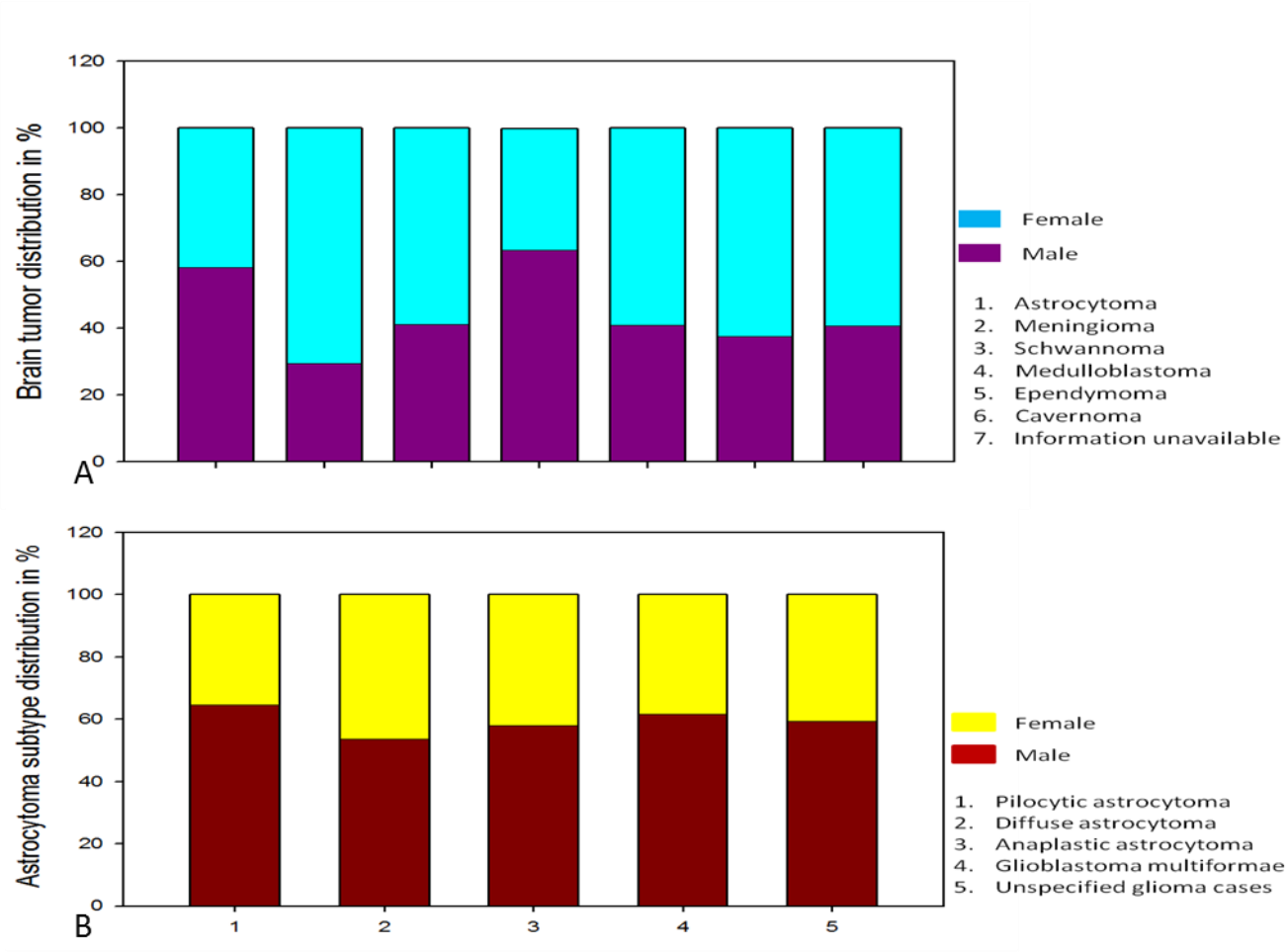

Fig.9: (A) Gender wise statistical distribution of brain tumors and (B) Astrocytoma sub types

Astrocytoma Type / sign or symptom	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	00	0.40%	0.40%	3.61%	5%	35%
Hearing impairment	0.80%	00%	1.20%	00%	2%	--
vision loss	1.20%	00%	0.80%	0.80%	3%	22%

Table 3: Most common symptoms associated with of astrocytoma subtypes.

[a]Krishna Institute of Medical Sciences registry .

[b]Data obtained from reference 10

2.4 Summary:

Cancer registration system is maintained in developed countries with classified information such as relative survival rate, annual incidence, risk factors as age of cancer development, etc. It can help to predict the survival outcome of patients with provided treatment, risk age groups, tumor location details which in turn can help in suggesting the effective therapeutic options.

National Cancer Institute, U.S.A has reported that the ethnic origin of population could influence mortality. This variance may be attributed to genetic alterations in population as well as to diagnostic accuracy. It may be the reason we cannot use the foreign statistical information improve therapeutic measures.

Here, we have observed the statistical distribution of brain tumors, with additional information as initial symptoms and median age of diagnosis. Further, tried to confront actual scenario of Indian population as compared to NCI and CBTRUS annual reports.

Our results displayed that in all brain tumor types, astrocytomas were most widespread with 53% followed by meningioma with 23%, schwannoma with 19%, medulloblastoma with 2%, ependymomas with 2% and cavernoma with 1% (Fig 7). Overall, brain tumors were more ubiquitous among females with 52% (Fig 9a and 9b). Among astrocytoma subtypes, diffuse astrocytomas was the most common with 38% followed by glioblastoma multiformae with 33% (Fig. 8). Amid 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 data from Indian population with that of from 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 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 year respectively. 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% .

Among astrocytoma subtypes, headache was 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% (Table 3). We have compared the data with from developed countries. Headache was noted with 56% as compared to 46% in Indian population. Seizures and vomitings were observed with 32% and 13% in population from developed countries. Deviation was observed among vision loss and memory loss symptoms. In developed countries, vision loss and memory loss was observed with 22% and 35% while our registry reported the same with only 3% and 5%.

The only inadequacy with our data may be that it is derived from single hospital and may not reflect the distribution of total population.

In conclusion, our results projects astrocytomas are highly reported neoplasms of central nervous system with middle aged group more prone. Among astrocytoma subtypes, diffuse astrocytoma was observed to be more prevalent followed by glioblastoma multiformae. Headache and seizures were frequently associated with high grade astrocytoma as compared to low grade tumors. Deviation was

observed in median age of diagnosis among glioblastoma multiformae and meningioma patients population when we compared the figures with that of patients with developed countries. In meningiomas, females were found to be more prone suggesting may be role of female sex hormones in tumor progression.

3.5 References:

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b. Prognostic significance of anatomic origin and glioblastoma with oligodendroglial component (GBMO) of astrocytoma patients'

2.6 Prognostic significance of anatomic origin and glioblastoma with oligodendroglial component (GBMO) of astrocytoma patients'

2.6.1 Introduction:

Astrocytoma are the most common malignancies of brain. World Health Organization (WHO) has classified astrocytoma in four grades on account of cellularity, nuclear polymorphism, mitotic index, microvascular proliferation and extent of necrosis [1]. Grade IV astrocytoma, also referred as glioblastoma multiformae, are the most aggressive primary tumors with worst prognosis and account for nearly 60% of malignant gliomas [2-4]. The overall prognosis for malignant glial tumors have not changed significantly since 1980 despite of advancements in course of diagnosis and mode of treatment [5]. Understanding prognostic factors affecting survival and clinico-pathological statistics can help to evaluate new measures for therapeutic intervention [6]. In India, we have previously reported lower median age and assessed symptoms associated with grade progression for astrocytoma patients [7]. Molecular markers such as PTEN, TP53, loss of heterozygosity on chromosome 10q and EGFR amplification have also been ascertained with poor prognosis [8, 9].

Anatomic origin of tumor is reported to contribute for prognosis. Tumors with origin on frontal lobe is reported to have better prognosis value than temporal, parietal or occipital lobe. Preferential location of low grade astrocytoma in certain areas is explained by functional, developmental or metabolic aspects. Type of surgery and location are reported as independent factors contributing to prognosis of glioblastoma multiformae. Patients' with tumor origin at frontal lobe have been shown to have progression free survival for one year than other locations [10-14]. Relative volume of glial tissue has been shown to influence development of gliomas in different anatomic sites. Functional differences between tissues in brain have been postulated for certain preferred locations of tumors [15]. Distinct molecular alterations prevalent in subset of glial tumors arising from different

anatomic origins have been reported [16-18]. However, there are very few studies evidencing the role of anatomic locations contributing to astrocytoma prognosis.

As described by World Health Organization, glioblastoma with oligodendroglial component (GBMO) have been described as high grade astrocytic neoplasms with discrete oligodendroglial differentiation areas displaying necrosis [1]. Certain studies have claimed that glioblastoma with oligodendroglial component display better prognostic pattern, yet this hypothesis remains controversial [19-24]. GBMOs have been reported to have distinct genetic alterations and clinical behavior and displays distinct IDH1 mutations along with 1p/19q co deletion [25, 26].

The aim of the present study was to screen clinical data on account of anatomic origin of tumor, to investigate the survival pattern reflected by tumor anatomy, and to analyze the overall survival among four grades of astrocytic tumors. We have also evaluated survival pattern of glioblastomas with oligodendroglial component and accessed overall survival of patients' of astrocytoma subtypes in our studied population..

2.7 Methods:

2.7.1 Selection of patients

The cases were reported at Krishna Institute of Medical Sciences (KIMS) Secunderabad, India during the time span of January 2009 to December 2014. Patients were approached after surgical resection. Each patient was assigned with unique ID. Informed consent was obtained from patients and each one was completely anonymized. Pathological distribution of tumor grade was determined by biopsy of surgical specimen at pathology department on account of cellularity, nuclear polymorphisms and MIB-1 staining. Registry of patients' was maintained with age/sex, grade of tumor and follow up details. All patients' were Indian citizens. In the subgroup of 40 patients with oligodendroglial component, paraffin embedded and H and E sections were used to confirm involvement of oligodendroglial component. These sections were found to contain round nucleus, cytoplasmic swelling and honeycomb appearance (Fig. 10)

Location of tumor was assigned roughly in four lobes on the basis of radiological reports: frontal, parietal, temporal and occipital (Fig. 12). All tumor samples were classified as per criterion specified by WHO into four groups: pilocytic astrocytoma, diffuse astrocytoma, anaplastic astrocytoma and glioblastoma multiformae. Among the studied population, subgroup of 40 glioblastoma patients' patients were selected with oligodendroglial component. These patients were not having previous history of low grade glioma. Oligodendroglial component was traced with H and E staining. Molecular marker details were available in few cases. Mainly, p53 and MIB-1 expression was evaluated. These patients were followed closely to obtain follow-up details (Table 4). Near total or subtotal resection was followed and confirmed by MRI imaging. Surgery was followed by radiotherapy and chemotherapy with temozolomide. MRI scan was practiced every 3 months to trace possible recurrence (Fig. 12).

Sr no.	Age/sex	Tumor location	MIB1	Necrosis	P53	Survival (in months)
1	55/M	Frontal lobe	+	+	+	15
2	59/M	Frontal lobe	+	+	+	11
3	29/M	Frontal lobe	+	+	--	13
4	42/F	Frontal lobe	+	+	+	10
5	60/F	Frontal lobe	+	+	--	28
6	50/F	Frontal lobe	+	+	+	26
7	16/M	Frontal lobe	+	+	--	31
8	63/M	Frontal lobe	--	+	+	26
9	48/M	Frontal lobe	+	+	n.a.	26
10	50/M	Frontal lobe	+	+	n.a.	21
11	25/F	Frontal lobe	+	-	+	n.a.
12	60/M	Frontal lobe	+	+	+	n.a,
13	25/F	Frontal lobe	+	+	--	18
14	51/F	Parietal lobe	+	+	+	9
15	27/M	Parietal lobe	+	+	n.a.	14
16	26/M	Parietal lobe	+	+	+	n.a
17	60/M	Parietal lobe	+	+	+	22
18	17 /F	Parietal lobe	+	+	--	1
19	43/M	Parietal lobe	+	+	+	22
20	33/M	Temporal lobe	+	n.a	n.a	54
21	30/M	Temporal lobe	+	+	+	1
22	63/M	Temporal lobe	+	+	n.a	1
23	57/M	Temporal lobe	+	+	+	2
24	60/F	Temporal lobe	+	-	+	n.a.
25	26/M	Temporal lobe	+	-	+	5
26	50/M	Temporal lobe	+	+	n.a	10
27	46/F	Temporal lobe	+	+	+	14
28	46/F	Temporal lobe	+	+	--	n.a.
29	55/M	Temporal lobe	+	+	n.a	23
30	54/M	Temporal lobe	+	+	+	31
31	22/F	Temporal lobe	+	+	n.a	17
32	25/M	Temporal lobe	+	+	n.a	7
33	53/M	Temporal lobe	+	+	+	20
34	52/M	Temporal lobe	+	+	--	15
35	55/F	Temporal lobe	+	+	+	14
36	41/M	Occipital lobe	n.a	+	--	9
37	45/M	Occipital lobe	+	n.a.	+	11
38	53/F	Occipital lobe	+	+	n.a.	n.a.
39	49/F	Occipital lobe	+	+	+	7
40	39/M	Occipital lobe	+	-	+	6

Table 4: clinical and molecular distribution of glioblastoma patients' with oligodendroglial component.

M=male, F= female, n.a. = details not available

In gliomas with involvement of two lobes (e.g. frontotemporal, predominantly located at frontal lobe), overlap was ignored and tumor location was designated at one location. In case of tumors

with overlap in several areas of brain, position was designated as per deeper anatomic site. Anterior location was given in same way with overlapping cases.

In total, our registry had 42 pilocytic astrocytoma cases, 181 diffuse astrocytoma cases, 78 anaplastic astrocytoma cases and 178 glioblastoma multiformae cases. Survival information was not available with total of 94 cases (Grade I= 09, Grade II= 50, Grade III = 10, Grade IV = 25). Out of 40 cases of glioblastoma with oligodendroglial component follow up details were available only with 36 cases. Almost all 40 cases were found with necrotic component.

2.8 Statistical analysis: Survival details were studied using Kaplan-Meier statistics to evaluate overall survival in pilocytic astrocytoma, diffuse astrocytoma, anaplastic astrocytoma, glioblastoma multiformae and glioblastoma with oligodendroglial component. Differences between survival groups were evaluated using Log rank (Mental cox) test. P value less than 0.05 was considered to be statistically significant.

2.9 Results:

Of all cases studied, 08.7% were of pilocytic astrocytoma, 37.7% diffuse astrocytoma, 16.2% anaplastic astrocytoma and 37.1% cases contributed glioblastoma multiformae. Histological diagnosis was used to confirm involvement of oligodendroglial component (Fig. 10). Glioblastomas with oligodendroglial component constituted 21% of total glioblastoma cases. Age of patients' was found to be in range of 16-63 years (male= 26, females=14). In previous retrospective studies, we have reported, 15.5 as median age for pilocytic astrocytoma, 54 for diffuse astrocytoma, 45 for anaplastic astrocytoma and 37.5 for glioblastoma multiformae in our tertiary experience. We also have reported nearly 60% of male (for grade I astrocytoma, male=33, female=18, grade II astrocytoma, male=124, female=107, grade III astrocytoma, male=51, female=37, and for glioblastoma multiformae, male=125, female=78) in the studied population [7].

In all astrocytoma cases we studied, 163 were located in frontal lobe, 57 in parietal lobe, 191 in temporal lobe, and 68 in occipital lobe (Table 5). Of glioblastoma with oligodendroglial component studied, 13 were in frontal lobe, 06 in parietal lobe, 15 in temporal lobe, 05 in occipital lobe. In pilocytic astrocytoma cases we studies, 03 were found to be located at frontal lobe, 01 at parietal lobe, 16 at temporal lobe and 22 at occipital lobe. In diffuse astrocytoma cases, 74 were found to be located at frontal lobe, 18 at parietal lobe, 74 at temporal lobe and 15 at occipital lobe. In anaplastic astrocytic tumors, 32 were found to be located at frontal lobe, 10 at parietal lobe, 27 at temporal lobe and 09 at occipital lobe. In glioblastoma multiformae cases, 54 were found to be located at frontal lobe, 28 at parietal lobe, 74 at temporal lobe and 22 at occipital lobe (Table 5).

Lobe location / Astrocytoma grade	Frontal	Parietal	Temporal	Occipital	Total
Grade I	3	1	16	22	42
Grade II	74	18	74	15	181
Grade III	32	10	27	9	78
Grade IV	54	28	74	22	178
Total tissues	163	57	191	68	479

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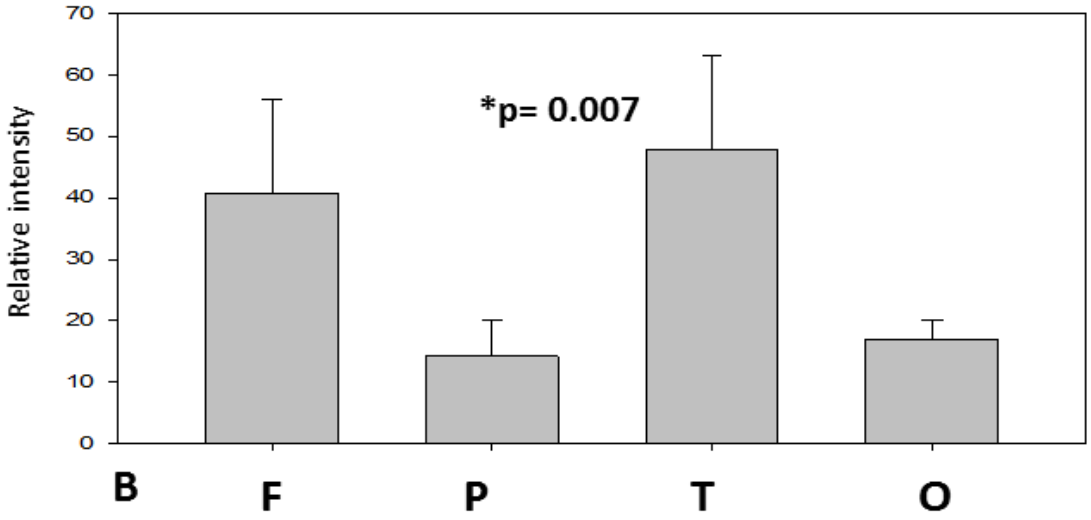


Table 5: Anatomic distribution of astrocytoma tissue samples. Tumors were found to follow non uniform distribution with dominance of frontal and temporal lobe (A and B). p= 0.007

F= frontal lobe, P= parietal lobe, T= temporal lobe, O= occipital lobe

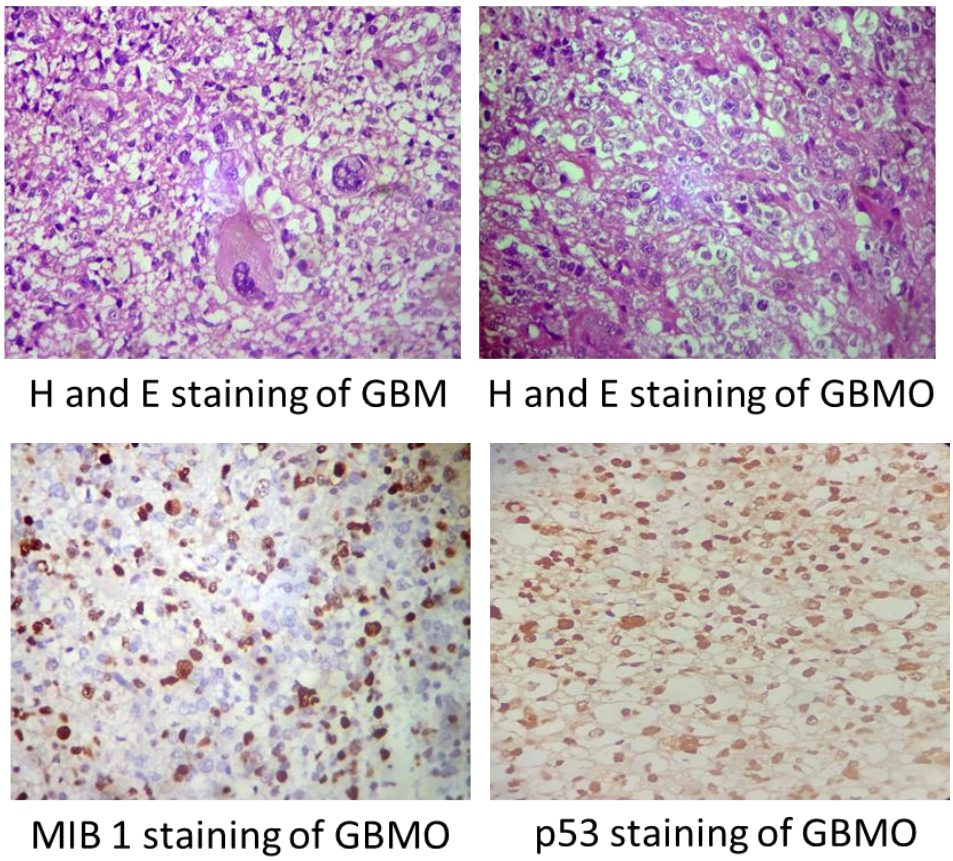


Fig.10: H&E staining of glioblastoma multiformae (GBM) and glioblastoma with oligodendroglial component (GBMO), MIB-1 and p53 Immunohistochemical staining of glioblastoma with oligodendroglial component (GBMO).

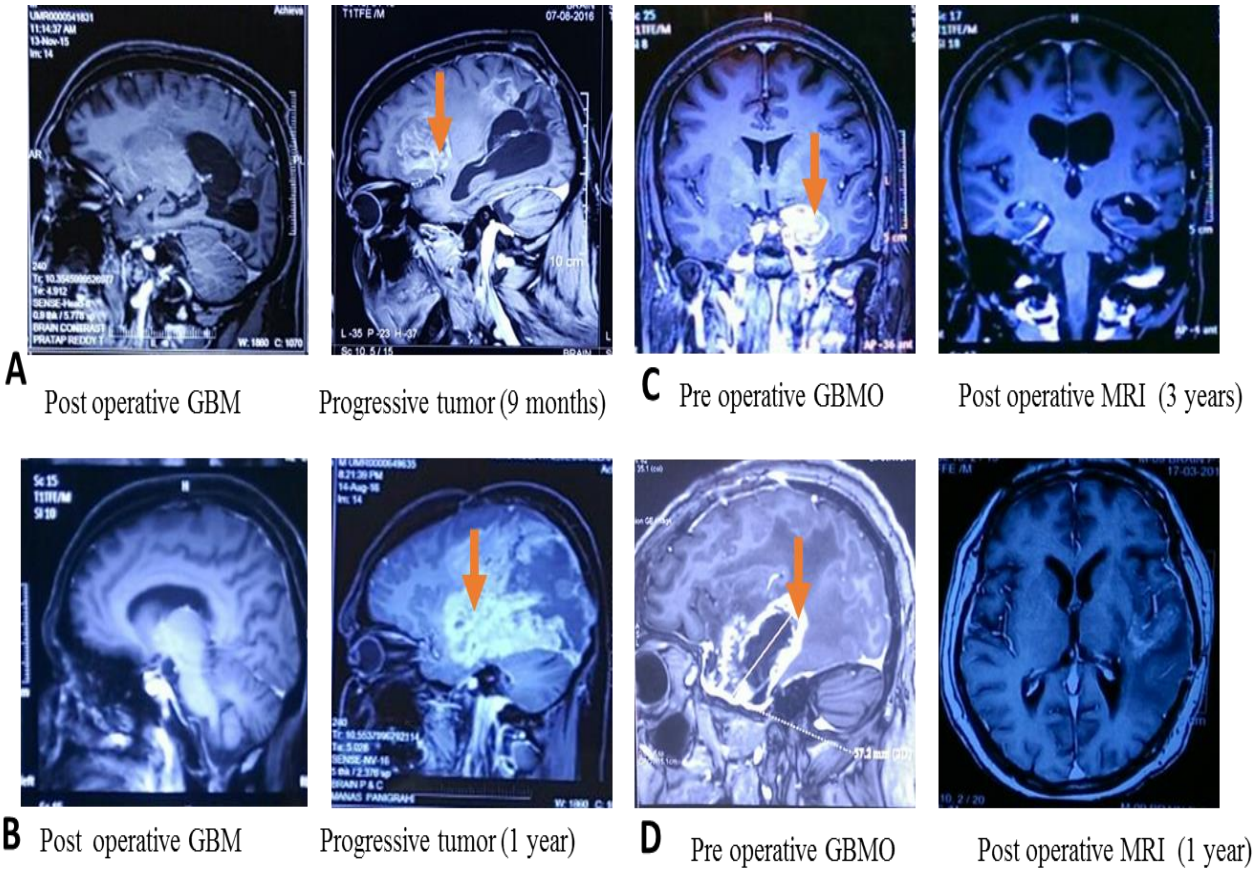


Fig. 11: Representative follow up MRI images of pre and post-operative glioblastoma and glioblastoma with oligodendroglial component. A: Post-operative glioblastoma with observed progression after 9 months of resection. B: Post-operative GBM with observed recurrence at occipital lobe after 1 year of resection. C: Pre-operative MRI scan image of occipital glioblastoma with oligodendroglial component and post-operative image taken after 3 years. D: Pre- operative MRI scan image of GBMO and post-operative MRI scan image taken after 1 year. Visible tumor has been marked by an arrow.

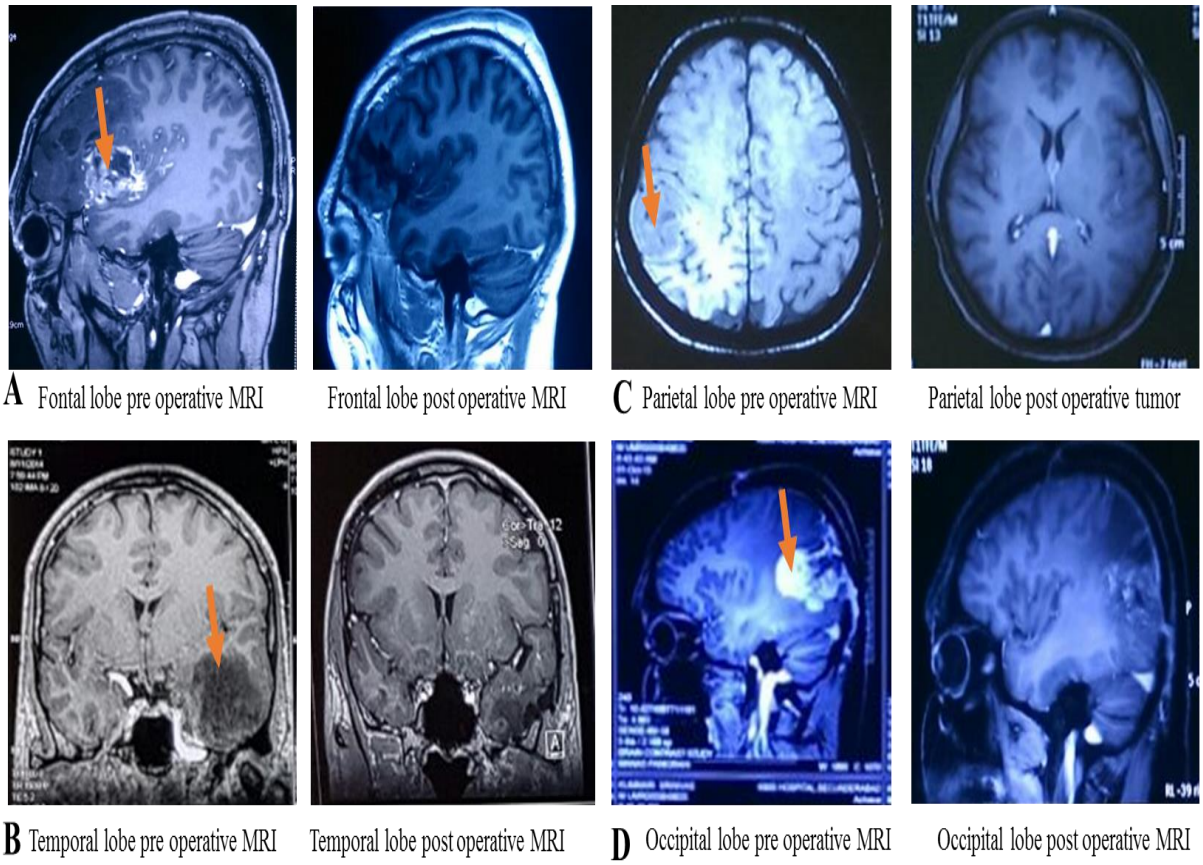


Fig. 12: Representative images showing MRI imaging based categorization of tumor location: pre and post-operative MRI images of tumor located at A: frontal lobe, B: temporal lobe, C: parietal lobe, D: occipital lobe. Visible tumor has been marked by an arrow.

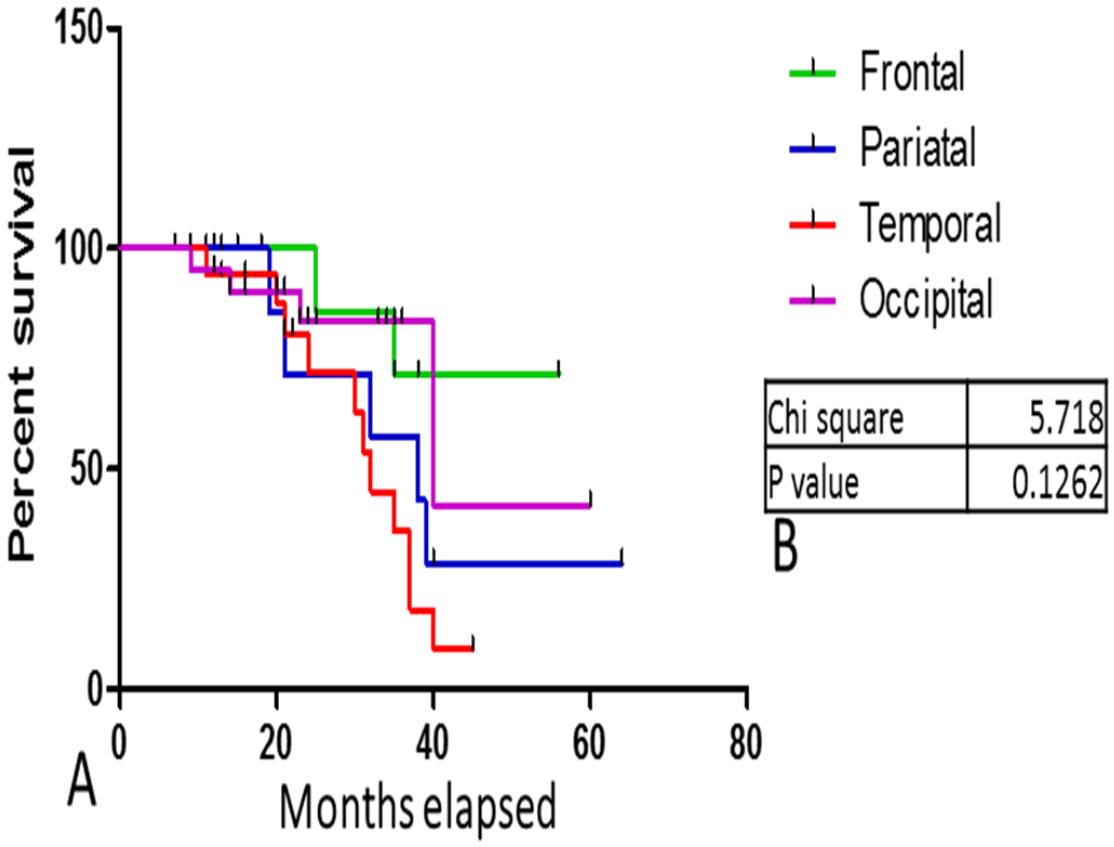


Fig. 13: Anatomic distribution based Kaplan-Meier survival of grade I astrocytoma patients' (A, n=42). Anatomic location was not significantly correlated with patients' survival (B, P= 0.12, Chi square = 05.7).

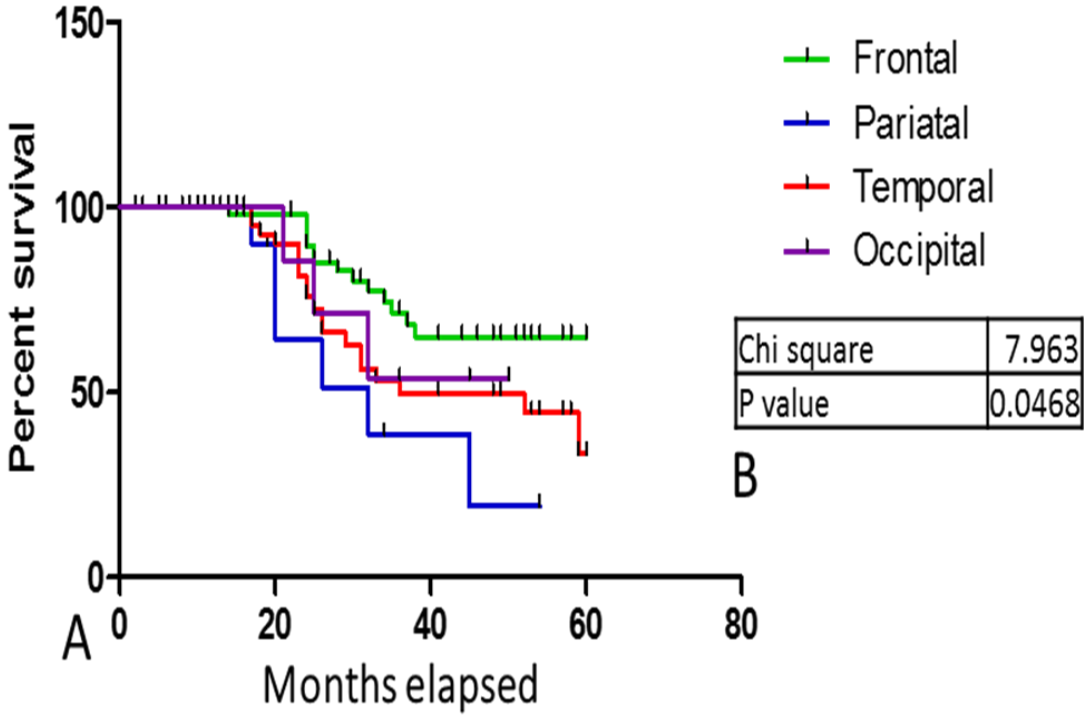


Fig. 14: Anatomic distribution based Kaplan-Meier survival of grade II astrocytoma patients' (A, n=181). Comparison of survival outcomes among four anatomic locations was statistically significant. (B, P= 0.04, Chi square = 7.9).

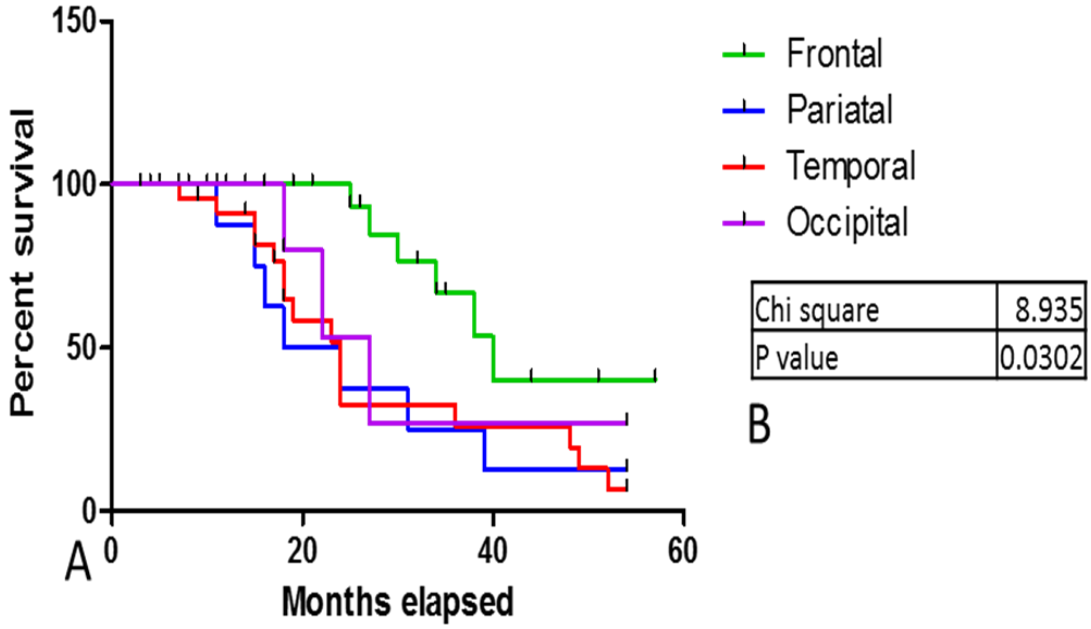


Fig. 15: Anatomic distribution based Kaplan-Meier survival of grade III astrocytoma patients' (A, n=78). Comparison of survival outcomes among four anatomic locations was statistically significant. (B, P= 0.03, Chi square = 8.9).

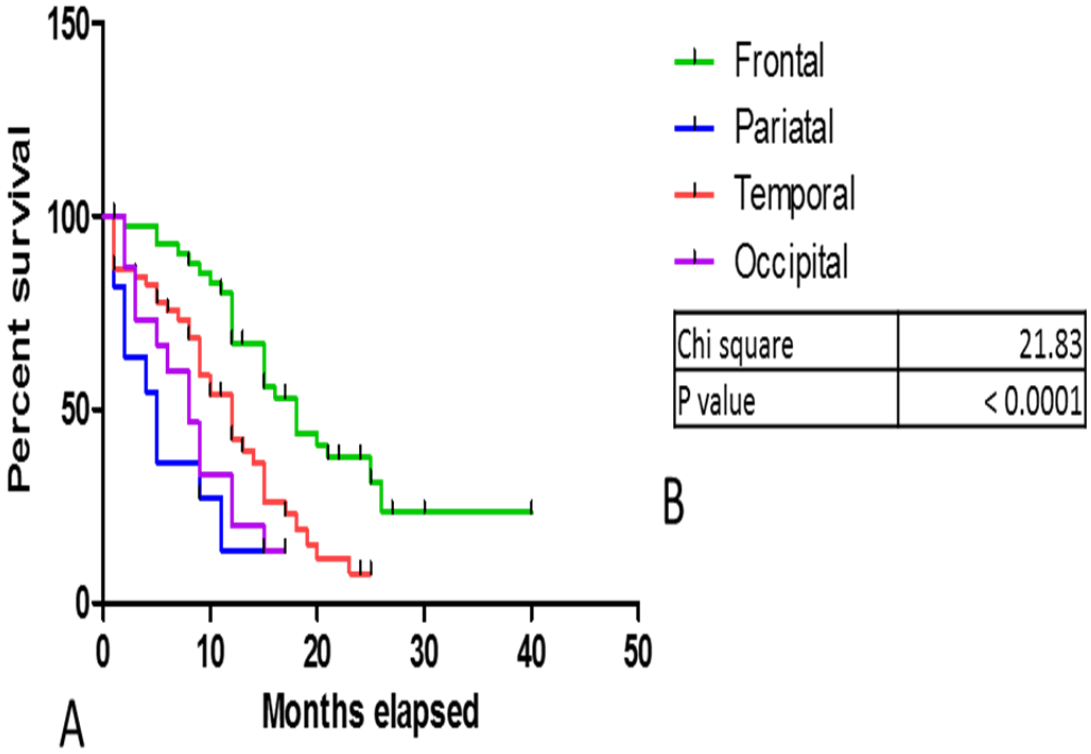


Fig. 16: Anatomic distribution based Kaplan-Meier survival curve of glioblastoma multiformae patients' (A, n=138). Survival of patients' with temporal, parietal and occipital origin of tumor was having poor survival outcome as compared to frontal lobe (B, P<0.0001, Chi square = 21.8).

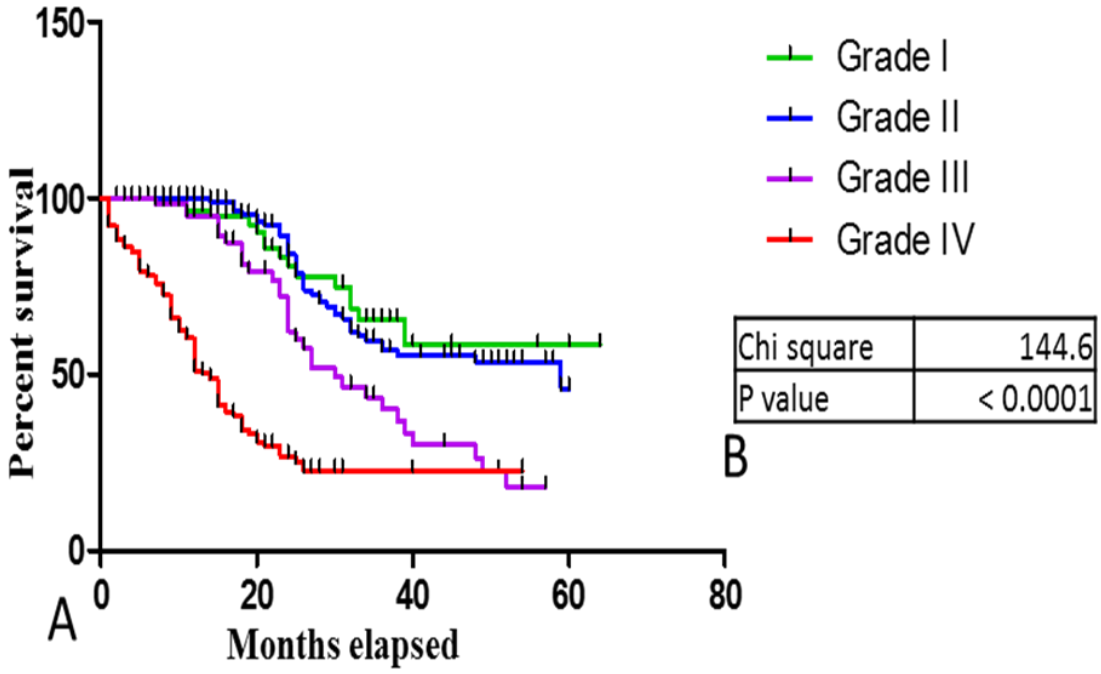
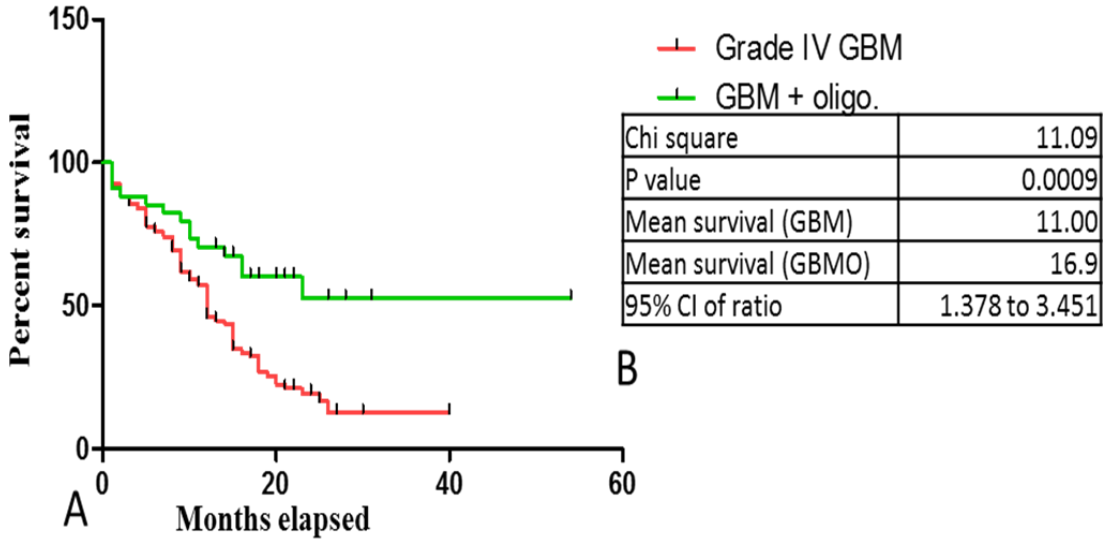


Fig. 17: Kaplan-Meier curve for survival of patients' of grade I, grade II, grade III and grade IV astrocytoma (A). Survival curve signifies poor survival in grade IV astrocytoma was statistically significant as compared to other three grades (B, $p<0.0001$).



Chi square	11.09
P value	0.0009
Mean survival (GBM)	11.00
Mean survival (GBMO)	16.9
95% CI of ratio	1.378 to 3.451

Fig. 18: Kaplan-Meier survival curve for patients with glioblastoma and glioblastoma with oligodendroglial component (A). Patients' having glioblastoma + oligodendroglial component (n=36) were having significantly longer survival than patients' with glioblastoma (n= 138) (B, p=0.0009).

We have also studied survival statistics for each grade of tumor based on their anatomic location (Fig 12). We found variation in survival of patients' on account of anatomic origin of tumor. Difference in anatomic location of tumor survival in Grade II (Fig. 14), Grade III (Fig. 15) and Grade IV (Fig. 16) was statistically significant. Here, survival in patients' with tumor not frontal lobe was found to be better than occipital or temporal lobe. Anatomic details were found to be not significant in case of grade I astrocytic tumors (Fig. 13). Overall, Grade I patients were having median survival value of 24 years, Grade II patients of 25 years, grade III patients of 19 years and grade IV patients of 12 years. Patients having glioblastoma with oligodendroglial components were found to have better survival than glioblastoma patients (Fig.18) with mean survival of 16.9 months

as compared 11 months of later. We have also analyzed survival of patients' in four astrocytoma subtypes (Fig. 17). Survival in patients with glioblastoma multiformae was worse than other astrocytoma subtypes (median survival= 12 months, mean survival= 11 months) and is found to be statistically significant ($p<0.0001$).

2.10 Summary:

The aim of present study was to clinically screen astrocytoma subtypes by their respective anatomic origin in brain and to study whether selective anatomic origin confers particular survival benefits. Further, we have prognostically evaluated role of oligodendroglial component in survival of glioblastoma multiformae patients. There have been few studies reporting involvement of oligodendroglial component in glioblastomas and is defined by specific molecular alterations [1, 25, 26]. Glioblastomas with oligodendroglioma is genetically heterogeneous and reported to represent 4% of glioblastoma multiformae. A study has reported LOH on 1p which is linked with better prognosis in anaplastic oligodendrogliomas and increased chemo sensitivity [27]. Other evidence however claim a higher incidence (17%) than later [28]. There are controversies about survival outcomes of glioblastoma with oligodendroglial component as some studies claim better survival for patients' with glioblastoma with oligo component [24, 26, 29, 30] while some studies acclaim no favorable prognostic values [20-22]. In our studies, out of total 178 cases, oligodendroglial component was confirmed in 40 cases (Table 4). This validates no frequent occurrence of glioblastomas with oligodendroglial component. We found glioblastoma patients with oligodendroglial component had better survival than previous one and is statistically significant ($p=0.0009$, Chi square= 11.09, 95% CI =1.37 to 3.4) (Fig. 18, Fig. 11). Glioblastoma patients were found with 11 mean survival months while GBM with oligo component patients were found to have 16.9 months of mean survival. We have studied total of 40 GBMO cases (Table 4). Survival details

were not available with 04 cases. We also found anatomic origin of tumor. In 40 cases, 13 found to be at frontal lobe, 06 at parietal lobe, 16 at temporal lobe and 04 at occipital lobe. Here, we observed non uniform distribution of tumor in brain mainly dominated by frontal and temporal lobe. Out of total subjects studied, 20 have been reported to survive for more than 2 years. In the studied population, we observed 50% of patients were older than 50 years at the time of diagnosis while previous studies [1, 31] claims this percentage to be more than 80%. We have also studied for MIB1 and p53 expression profile in the studied population. Out of 40 GBMO cases, 22 were found to be positive with p53 overexpression while 4 cases were negative. Details were not available with 10 cases. Overall, we found p53 positivity in 55% of cases. Recent reports claims p53 positivity in more than 70% of cases and is associated with IDH1 mutations [26, 32]. Of total 40 GBMO cases, 38 (95%) were found to be positive for MIB1 staining while other reports claims this positivity to be as low as 31% [33] and do not possess prognostic significance [34]. We found necrosis to be prevalent in 34 (85%) cases. 2 cases were found to be negative while data was not available for 4 cases. Previous reports supports for presence of necrosis in GBMO and has been correlated with less favorable prognosis in anaplastic oligoastrocytoma [30, 35]. Oligoastrocytic tumors with necrosis have been designated as glioblastomas with oligodendroglial component by WHO 2007 classification[1]. On pathological scale, GBMOs are defined as anaplastic astrocytic tumors with necrosis [36, 37] and develops from malignant transformation of low grade glial tumors. So, a combined approach to look for associated genetic mutations is necessary.

We have further studied the anatomic location in astrocytomas and found dominated occurrence at frontal and parietal lobe. Overall, we found tumor anatomic origin at frontal lobe in 34% of cases, at parietal lobe in 11.8% of cases, at temporal lobe in 39.8% of cases and at occipital lobe in 14.1% of cases (Table: 5, p=0.007). While, previous reports claim frontal lobe as anatomic origin for 43% gliomas, parietal lobe for 25% of cases, temporal lobe for 28% of cases and occipital lobe for only

3% of cases [13]. Prominent origin at frontal and temporal lobe found in our findings are consistent with existing literature [15]. Studies have shown that tumor occurs more frequent involvement of right hemisphere in brain [38]. These reports, however does not comment on prognostic significance of anatomic origin. We show for the first time that tumors with frontal lobe as anatomic origin have better prognostic significance than temporal, parietal and occipital lobe. In grade II (Fig. 14) ($p=0.04$) and grade III (Fig. 15) ($p=0.03$) astrocytoma, better survival at frontal lobe was statistically significant as compared to parietal and temporal lobes. In glioblastoma multiformae, temporal, parietal and occipital lobe as anatomic origin was correlated with poor survival outcomes than frontal lobe (Fig. 16) ($p<0.001$). A recent study has shown that tumors located in right hemisphere are correlated with worst prognosis [39]. Further, it was observed that tumors in this region have larger volume leading to extensive infiltration and tend to difficult to resect [39, 40]. Previously, tumors located at subventricular zone (adjacent to right parietal location) are correlated with worse prognosis. These studies were based on qualitative assessment of tumor location [41, 42]. The association of poor prognosis at parietal, temporal and occipital lobes may be related to underlying tumor biology and neuroanatomy at those particular locations. Along with it further investigations are necessary to understand the molecular mutations associated with particular locations to know more about origin of tumors and associated pathology. Overall, our findings highlight frontal lobe was associated with significantly better prognostic values in anaplastic astrocytoma and in glioblastoma multiformae.

We have further studied retrospectively pattern of survival in patients with astrocytoma. We found that median age of survival (in months) 24 for pilocytic astrocytoma, 25 for diffuse astrocytoma, 19 for anaplastic astrocytoma and 12 for glioblastoma multiformae. A recent report claims survival of patients with glioblastoma multiformae improved to be 10.3% in 2009-10 than 4.4 % in year 1999-2000 [43]. However, in latest 5 year period, we found 9% of patients' live beyond 2 years. In 41% of

cases, patients succumbed to glioblastoma within one year while 48% in second year. Our data represents poor survival of glioblastoma patients beyond two years (Fig. 17).

Cancer registry system in developed countries and a developing nation like India differs mainly on ground of uniform collection and reporting of clinico-pathological data. The dedicated cancer registry there helps to estimate accurate information about statistical distribution and mortality rate in a population [44]. While in India, epidemiological and clinico-pathological studies are reported as tertiary experiences [7]. Our study has several advantages, it is for the first time in India we are reporting survival information in a set of population. Second, we report our data with wide participation of patients and represent a dataset with significant coverage.

In conclusion, our findings indicates frontal and parietal lobe as prominent anatomic origin of tumors. Frontal lobe as anatomic origin was correlated with better prognosis while temporal, parietal and occipital origin was significantly associated with worse clinical outcomes in high grade astrocytoma patients'. Further, glioblastoma with oligodendroglial component was associated with better survival with overexpression of p53 and MIB-1. Among astrocytoma subtypes, glioblastoma multiformae was found with worse survival outcomes with mean survival age of 11 months.

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Chapter III

Expression and significance of CD200R1 and SIRP Alpha in astrocytoma progression.

Introduction

3.1 Introduction:

3.1.1 Biology of CD200R:

CD200R1 and SIRP Alpha belong to the paired receptor family of proteins. They have closely related extracellular domains but differ in transmembrane and cytoplasmic regions [1]. They are expressed on natural killer cells and cells of myeloid origin [2]. CD200-CD200R interaction shares similarity with CD47- SIRP Alpha signaling. In addition to its distribution mainly on myeloid cells, CD200 and CD47 are viewed as therapeutic targets for leukemia [3, 4].

CD200 was previously known as orphan receptor 2 (OX-2). Immunomodulatory proprieties of CD200 were further uncovered upon identification of its receptor, CD200R. OX-2 was conferred with cluster of differentiation number 200 by the 7th Workshop and Conference on Human Differentiation Antigens organized in the year 2000 [5]. CD200R is reported to be highly glycosylated protein with molecular weight ranging from 60-100 kD, mainly depending on the two criterions: the extent of glycosylation and the expression cell type [5]. The functional aspects of CD200-CD200R interaction were shown with CD200R blocking antibody. CD200 and CD200R shares a sequence homology with two IgSF domains, a cytoplasmic tail and a transmembrane region. Cytoplasmic tail of CD200R is larger than CD200 [6]. CD200-CD200R interaction play an important role in inflammatory diseases and tissue inflammations [7, 8]. Engagement of CD200 with CD200R is shown to drive anti-inflammatory signals by CD200R and the response dependence of CD200R with CD200 is also noted [9, 10]. CD200-CD200R interaction has been shown to inhibit the activation of ERK, JNK and p38 mitogen activated protein kinase (MAPK) pathway [9]. Importance of CD200 has been noted on number of cancers. CD200 overexpression has been positively correlated with aggravated metastasis in melanomas [11] while in multiple myeloma, acute myeloid leukemia, chronic leukemia of B cells and lymphoid malignancies, CD200 overexpression is

correlated with poor prognostic outcome [12-16]. Blockade of CD200 pathway is postulated to restore the anti-tumor responses [17]. Recent studies in hepatocellular carcinoma projects CD200R as prognostic factor and a potential drug target [18]. Studies in breast cancer show that absence of CD200R1 expression leads to increased release of inflammatory cytokines such as TNF-alpha and IL-6 and decreased tumor infiltrating cytotoxic T cells [18]. Although CD200 has been documented to have tumor promoting role in leukemia [15, 16], there are conflicting evidences regarding its role in solid tumors. In squamous cell carcinoma, increased CD200 expression was linked with increased metastatic survival [20] while associated with tumor initiating proprieties in human basal carcinoma [21]. On the other hand, decreased CD200 expression on melanocytes resulted in decreased lung metastasis. Agonistic monoclonal antibody mediated CD200R expression is associated with decreased tumor formation of CD200- negative melanoma in lungs. The conflicting evidences about role of CD200R may be attributed to bidirectional immune activation in cancer progression and metastasis.

The role of CD200R1 in astrocytoma biology is not completely understood. Here, we have shown that CD200R1 is uniquely over expressed in low grade (GII) tumor tissue samples and its expression was decreased with grade progression.

3.1.2 Biology of SIRP alpha:

Signal regulatory protein alpha (SIRP Alpha or CD172a) is a membrane protein mainly expressed on myeloid cells and macrophages. SIRP alpha mediates its signaling in association with SHP-1 and SHP-2 phosphatase. Structurally, SIRP alpha contains three Ig like domains, a transmembrane domain and a cytoplasmic tail with four tyrosine residues [22]. CD-47 was identifies as ligand associated with SIRP alpha in human [23], mouse [24] and rat [25]. CD-47 is widely reported to be expressed on brain tumors [26], ovarian cancer [27] and breast cancer [28]. In brain and ovarian cancer studies [26, 27], CD-47 has been reported to interfere with phagocytosis axis and modulate the tumor progression. In breast cancer, studies have shown that CD-47 signaling is regulated by TNF-NFKB1 axis by directly interacting with its enhancer.

The SIRP alpha- CD-47 interaction is involved in controlling phagocytosis. CD-47 is termed to display 'don't eat me' signal through its interaction with SIRP alpha [39, 30]. The interaction is postulated to have role in immune evasion by inhibiting the phagocytosis. SIRP alpha is postulated to play a key role in regulation of dendritic cell activity and its therapeutic abrogation may enhance the efficacy of dendritic cells based tumor vaccines [31].

Studies have shown that SIRP Alpha mRNA is expressed in astrocytoma cell lines as well as in paraffin embedded glioblastoma tissue sections [13], however these findings are not supported with relevant control brain tissue samples. Cloning and overexpression experiments in U87MG reveal that SIRP Alpha modulates signaling pathways in EGFR dependent ways [14].

We primarily show that SIRP Alpha mRNA is overexpressed from control brain to GII astrocytoma tissues and further under expressed in high grade (GIII and GIV) tissue samples. However, we could not detect protein expression in low and high grade tumor tissue samples. We hypothesize post transcriptional loop pertinent in the form of miRNAs in regulation of SIRP Alpha expression.

Computational prediction accustoms presumptive involvement of hsa-miR-520d-5p and hsa-miR-520d-3p in regulation of SIRP Alpha expression. These miRNAs were subsequently found to follow the expression pattern similar to SIRP Alpha transcripts.

Materials and methods

3.2 Materials and methods

3.2.1 Sample collection and processing:

Surgically resected astrocytoma tissues were retrieved from Krishna Institute of Medical Sciences (KIMS), Secunderabad, India. Tissues were clinically and histopathologically diagnosed in different pathological grades at pathology department of same hospital. Tissues were immediately snap frozen in liquid nitrogen and further stored in -80^{0}C. A part of surgically resected tumor tissue was used for RNA isolation and western blotting and other part was used for immunohistochemistry studies. Classification of samples was in accordance with WHO [32]. Epilepsy tissue samples were used as control. Further, two normal brain and 6 epilepsy tissue were collected from National Institute of Mental Health and Neurosciences (NIMHANS), Bengaluru, India. Informed consent was obtained from patients'. This study was approved by institutional ethics committee. All the participants were completely anonymized.

3.2.2 RNA isolation and real time PCR:

Total RNA was isolated by Trizol reagent (Sigma, cat. No: T9424) according to manufacturer's instructions. Isolated RNA was quantified using Nano drop spectrophotometer (Thermo scientific, USA). Same pool of RNA was used to evaluate SIRP Alpha transcripts and miR-520d-3p and miR-520d-5p. GAPDH was used as internal control for SIRP Alpha expression and U6 as internal control for micro RNA analysis. Primers sequence used were:

SIRP Alpha forward primer:5'-TACAAGGTTGCATGAGCCCGSIRP-3'

Alpha reverse primer: 5'-TGGCATACTCCGTGTGGTTGGAPDH-3'

GAPDH forward primer: 5'-AAGGCTGGGGCTCATTTGCAG-3'

GAPDH reverse primer: 5'- GCAGGAGGCATTGCTGATGATC-3'

U6 forward primer:5'-GCTTCGGCAGCACATATACTA-3'

U6 reverse primer: 5'-GGAACGCTTCACGAATTTGC-3'

Hsa-miR-520d-5p RT primer: 5' -
GTTGGCTCTGGTGCAGGGTCCGAGGTATTCGCACCAGAGCCAACGAAAGG – 3',

Hsa-miR-520d-5p mature form forward primer: 5' - GTTGGCTACAAAGGGAAGC - 3'

Hsa-miR-520d-3p RT primer: 5'-
GTTGGCTCTGGTGCAGGGTCCGAGGTATTCGCACCAGAGCCAACACCCAC – 3'

Hsa-miR-520d-5p matured form forward primer: 5' - GTTTGGAAAGTGCTTCTCTTTG - 3',
universal reverse primer: 5' - GTGCAGGGTCCGAGGT - 3'.

CD200R1 forward primer: 5'- GAGCTACTTCCTGTTCCAGG-3',

CD200R1 reverse primer: 5'- GGCTGCATTTCATCCTCCTC-3

C-DNA synthesis for SIRP alpha, CD200R and internal controls was performed using PrimeScript 1st strand C-DNA synthesis kit (Takara, cat. No 6110A). Manufacturer's instructions were followed for reaction setup.

For C-DNA synthesis of microRNA, equal quantity of RNA as used for accessing mature form expression. Initially, pulsed RT protocol (65^0C-5minutes, 16^0C-30 minutes [30^0C -30seconds] × 60 times, 42^0C -30seconds, 50^0C-10 seconds, 85^0C-5 minutes) was followed for C-DNA synthesis. SYBR green (Takara, cat no: RR041) reagents were used for real time PCR of SIRP Alpha and miRNAs. Analysis was done by 2–ΔΔCT method [33]. The efficiency and sensitivity of present protocol has been discussed in previous literature [34-36]

3.2.3 Western blotting and imaging:

100 mg of tumor and control brain tissue was homogenized in RIPA buffer supplemented with protease and phosphate inhibitor cocktail (Sigma, cat no.: P8340). Protein concentration was estimated using Bradford reagent (Sigma, cat. No.; B6916). 75 micrograms of estimated protein was mixed with 6x gel loading dye. After boiling for 5 minutes on water bath, protein was subjected for 10% SDS polyacrylamide gel electrophoresis followed by western blotting. Proteins in SDS gel were transferred to nitrocellulose membrane (Millipore) at 30 volts for 12 hours overnight at 4^0 C. SIRP Alpha primary antibody (Santa Cruz, Cat no: sc-376884) was diluted to 1:500 in 5% skim milk solution incubated at 4^0C for 8 hours followed by anti-mouse secondary antibody (Sigma, cat no. A9044) for one hour at room temperature. CD200R1 primary antibody (Santa Cruz Biotechnology) was diluted to 1:500 in skim milk solution and incubated overnight at 4^0C. Prior to this, blots were blocked with 5% skim milk powder in TBST. Rabbit anti-goat secondary antibody (ab6741) was used with 1:10000 dilution and incubated for 1hrr at room temperature. This was followed by intermittent TBS washes.

SIRP Alpha and CD200R protein expression was normalized with Beta actin primary antibody (Sigma, cat no. SAB1305567) with 1:5000 dilution. Western blots were imaged using VersaDoc (Bio-Rad, USA) molecular imager.

3.2.4 Immunohistochemistry analysis:

Paraffin embedded sections of 5 micron thickness were used for immunohistochemistry (IHC) staining. Initially, sections were deparaffinized by heating at 100^0C on slide warmer followed by series of xylene and ethyl alcohol washes. Antigen retrieval was performed with sodium citrate buffer (10mM sodium citrate, 0.05% of Tween20, pH 6.0) in microwave oven for 6, 3, 3, 3 minutes. SIRP Alpha and CD200Rprimary antibody with 1:200 dilution was used. Further steps were followed as per user manual of Invitrogen IHC detection kit (cat no: 87-9673).

3.2.5 Computational prediction of miRNAs targeting SIRP Alpha transcript:

Micro RNAs (miRNA) putatively targeting to 3' and/or 5' UTR of SIRP Alpha were screened by use of four tools as target scan (http://www.targetscan.org/) miRanda (http://www.microrna.org/microrna/home.do), miRWalk (http://zmf.umm.uni-heidelberg.de/apps/zmf/mirwalk2/), miRo (http://ferrolab.dmi.unict.it/miro). miRNAs collectively predicted by four of these programs were selected for further analysis. Candidate miRNAs were then screened for SIRP Alpha 3' and 5' UTR binding efficiency by RNA hybrid web server (http://bibiserv.techfak.uni-bielefeld.de/rnahybrid?id=rnahybrid_view_submission).

3.4 Statistical analysis:

Statistical analysis were performed using SigmaPlot version 11.0. All results were expressed as mean + SD. Statistical significance between groups was analyzed by one way ANNOVA. P value <0.05 was considered as statistically significant.

Results

3.5 Results: CD200R1:

3.5.1 Real time PCR expression profile shows CD200R1 is significantly expressed in low grade astrocytoma tissues (GII):

Real time expression profile shows that CD200R1 transcripts were overexpressed in low grade (GII) and high grade (GIII and GIV) astrocytoma tissues. Decreased transcripts expression in high grade tumor tissues were statistically significant as compared with expression in low grade (GII) tumor tissues ($p<0.05$). We observe transcript expression was increased from control lo low grade and further decreased with grade progression. CD200R1 transcript expression was normalized with GAPDH (Fig. 19).

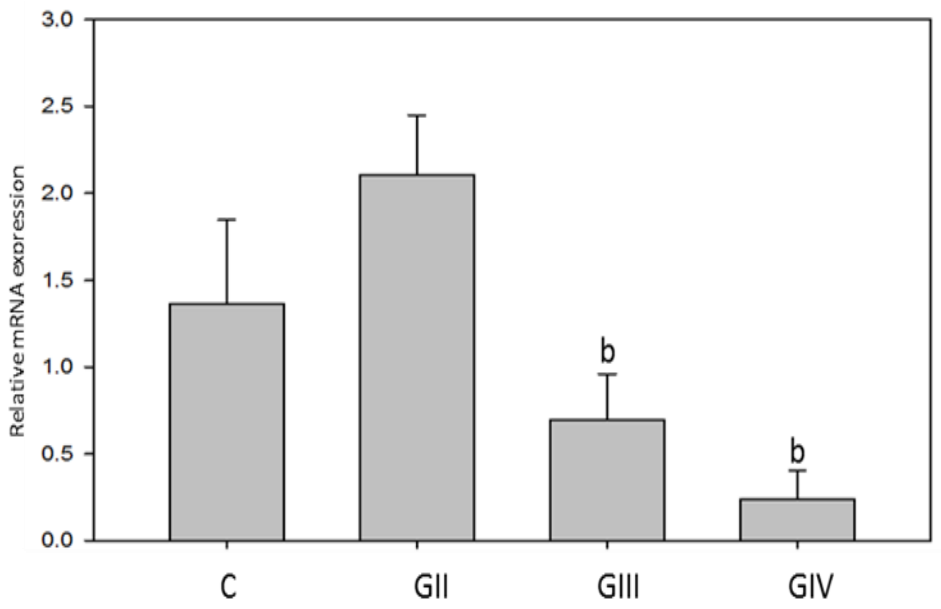


Fig. 19: Real time expression profile of CD200R1 in control, low (GII) and high grade (GIII and GIV) of astrocytoma tissue samples. Overexpression observed in GII tissue samples was statistically significant as compared to expression in high grade tissues ($p< 0.05$). GAPDH was used as internal control.

3.5.2 CD200R1 protein was overexpressed in low grade tumor tissue samples (n=23):

Western blotting experiment (Fig. 20 A and B) shows that CD200R1 protein followed the transcript expression pattern. Initially, protein expression was found to be increased from control (n=04) to low grade (n=06) progression and observed subsequent decrease with further higher grades. Densitometric expression analysis (Fig. 20: B) shows decreased expression in high grade tumor tissues was statistically significant as compared with low grade tissues. CD200R1 protein expression was normalized with beta actin protein expression.

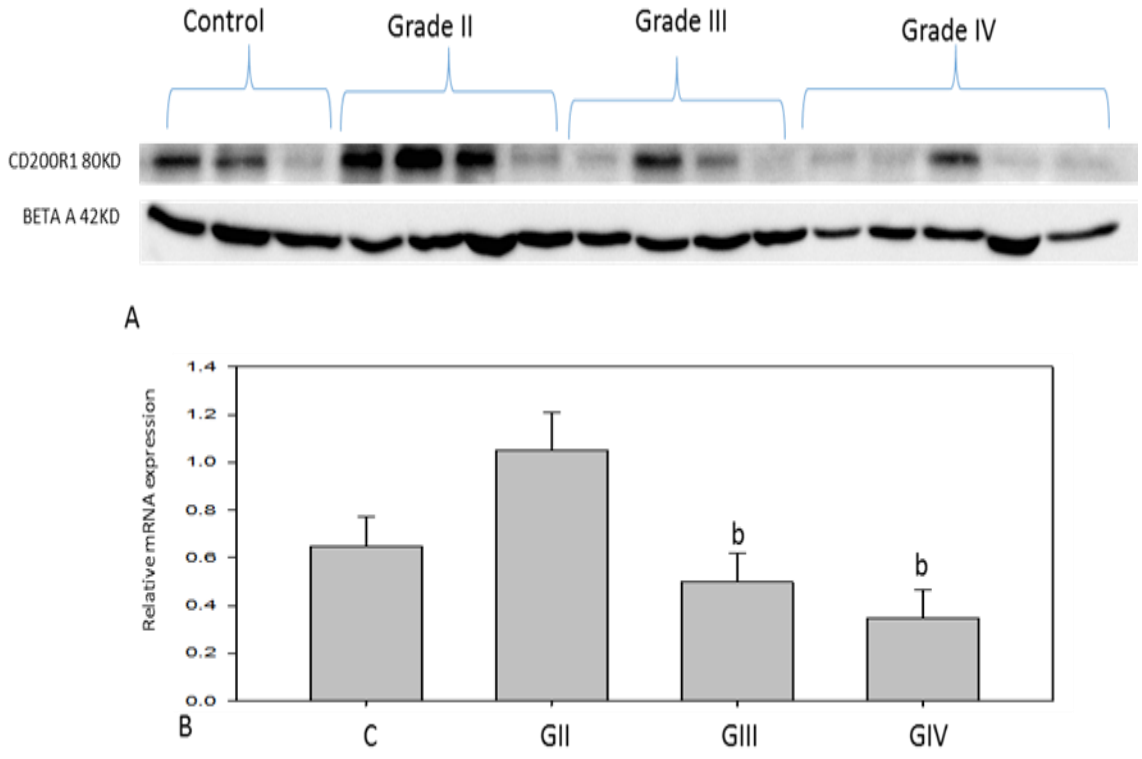


Fig.20: Western blotting (A) and densitometric analysis (B) for CD200R1 protein expression in control, low grade (GII) and high grade (GIII and GIV) astrocytoma tissue samples. Decreased expression of CD200R1 protein in high grade tissue samples was found to be statistically significant

as compared with low grade tumor tissue samples ($p<0.05$). Beta actin was used as internal control. Immunohistochemistry analysis of CD200R1 expression in control, low grade and high grade (GIV) tumor tissue samples:

Immunohistochemistry results (Fig. 21) further supplemented western blotting outcome. GII tissue sections were found to be intensely stained while GIV tissue were mostly found negative for CD200R1 protein expression. Control brain tissues were moderately stained.

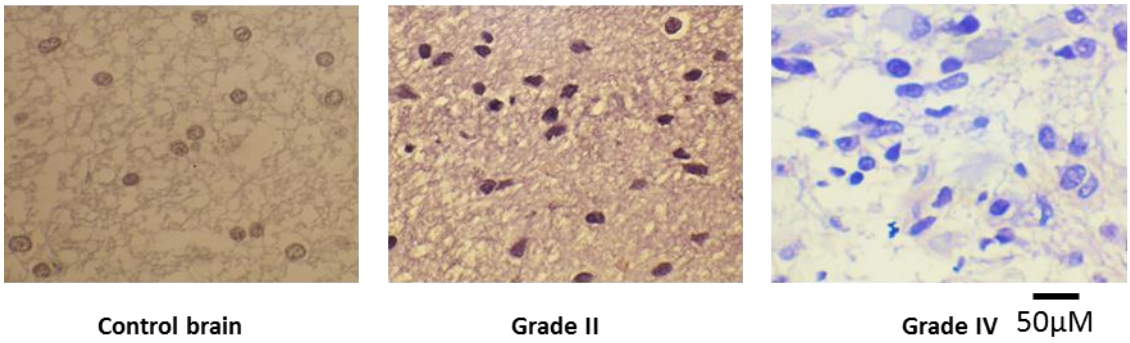


Fig.21: Immunohistochemistry analysis for CD200R1 in control brain, low grade (GII) and high grade (GIV) astrocytoma tumor tissue sections.

3.5.3 Correlation of CD200R1 protein expression with clinico-pathological parameters:

Overall, CD200R1 expression pattern was evaluated in 19 surgically resected human astrocytoma tissue samples and 04 control brain tissues. We found that CD200R1 expression was not correlated with age ($p=0.196$) and sex ($p=0.416$) of patients. Out of total cases we studied, 10 patients' were found to have age below or equal to 45 while 13 were found to have age above 45. This study constituted 14 male and 09 female participants. 5/6 low grade tissues were found to express CD200R1 protein while 03/06 GIII and 02/07 tissues were positive with CD200R1 expression. Out of 4 control brain tissues, only one was found to be negative for CD200R protein expression (Table 5).

Parameters	No of cases	CD200R1 protein Expression		P Value
		Positive	Negative	
All cases	23			0.196
Age				
<=45	10	06	04	
>45	13	07	06	
Gender				0.416
Male	14	08	06	
female	09	05	04	
WHO Grade				<0.05
II	06	05	01	
III	06	03	03	
IV	07	02	05	
Control	04	03	01	

Table 5: Clinico-pathological evaluation of patients in light of CD200R1 protein expression.

3.6 Results- II: SIRP alpha:

3.6.1 SIRP Alpha mRNA expression was confirmed by real time PCR:

SIRP Alpha mRNA expression was investigated in 53 tissue specimens. Normal brain and epilepsy tissue samples were used as control. Low grade tumor tissue (GII) displayed increased expression than control brain and high grade (GIII and GIV) tissue samples ($p< 0.05$). Expression of SIRP Alpha transcripts were found to be decreased with subsequent grade progression (Fig 22). We have further analyzed expression of miR-520d-5p and miR-520d-3p in astrocytoma tissue samples.

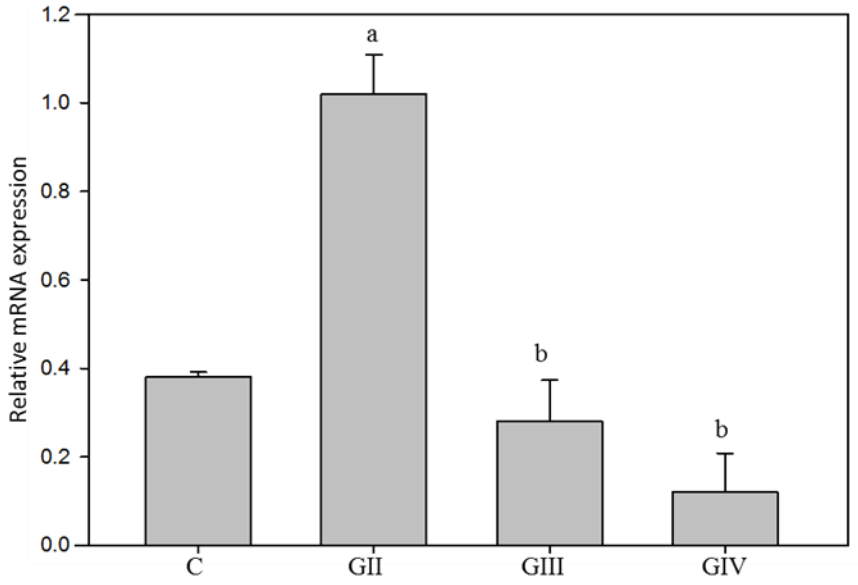


Fig. 22: Real time PCR expression of SIRP Alpha in control, GII, GIII and GIV tumor tissue samples. Rate of increased transcript expression in GII was statistically significant as compared with control, GIII and GIV. $P<0.05$.

miR-520d-5p and miR-520d-3p mature form expression were detected by real time PCR:

mir-520d-3p (Fig. 2) and miR-520d-5p (Fig.24) expression was evaluated by real time PCR. Initially, miR-520d-5p and miR-520d-3p expression was found to be upregulated from control to GII

progression and decreased in higher grade tissue samples. Transcript expression increase of miR-520d-3p and miR-520d-5p in GII was statistically significant as compared with control, GIII and GIV.

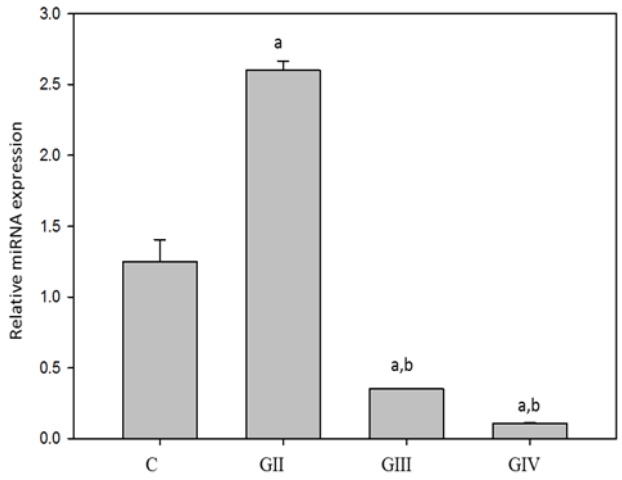


Fig. 23: Real time PCR expression profile of Hsa-miR-520d-3P in control and three astrocytoma grades. Increase in GII was statistically significant as compared with control, GIII and GIV ($p < 0.05$). U6 RNA was used as internal control.

C= control brain, GII= grade two, GIII= grade three, GIV=grade four.

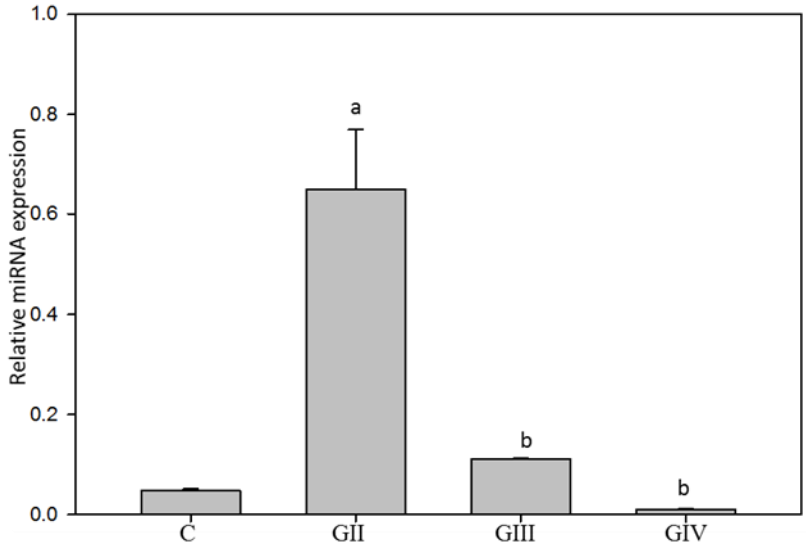


Fig.24: Real time PCR expression profile of Hsa-miR-520d-5P in control and three astrocytoma grades. Increase in GII was statistically significant as compared with control, GIII and GIV ($p < 0.05$). U6 RNA was used as internal control.

C= control brain, GII= grade two, GIII= grade three, GIV=grade four.

3.6.2 miR-520d-5p and miR-520d-3p mature form expression were detected by stem-loop real time PCR:

Mir-520d-3p (Fig. 2) and miR-520d-5p (Fig.3) expression was evaluated by stem-loop real time PCR. Initially, miR-520d-5p and miR-520d-3p expression was found to be increased from control to GII progression and subsequently decreased in higher grade tissue samples. Elevated mature form expression of miR-520d-3p and miR-520d-5p in GII was statistically significant as compared with control, GIII and GIV.

3.6.3 SIRP Alpha protein expression in control, low and high grade astrocytoma tissue samples (n=53):

SIRP Alpha protein expression pattern was evaluated in control (n=14), low grade (GII, n=11) and high grade (GIII, n=9 and GIV, n=19) tissue samples. Unlike its mRNA expression pattern, we did not find protein expression in low and high grade tissue samples (Fig 25, Fig.26, Fig. 27, 28 and Fig.29). Western blotting results were supplemented by immunohistochemistry analysis. Control brain sections showed staining while low grade and high grade sections were stained negative.

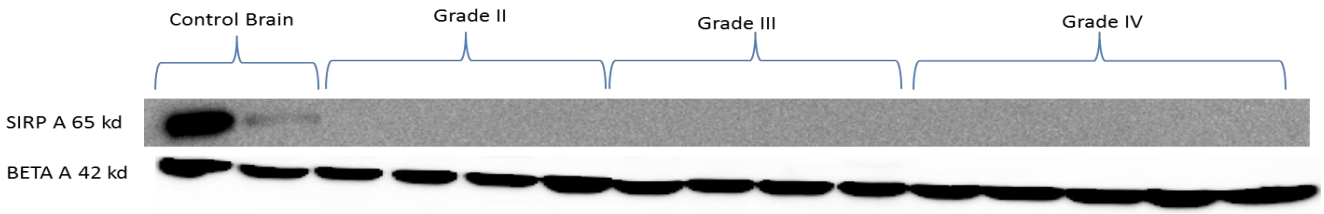


Fig. 25: Western blot for SIRP Alpha in control, low (GII) and high grade (GIII and GIV) tissue samples. Beta actin was used as internal control.

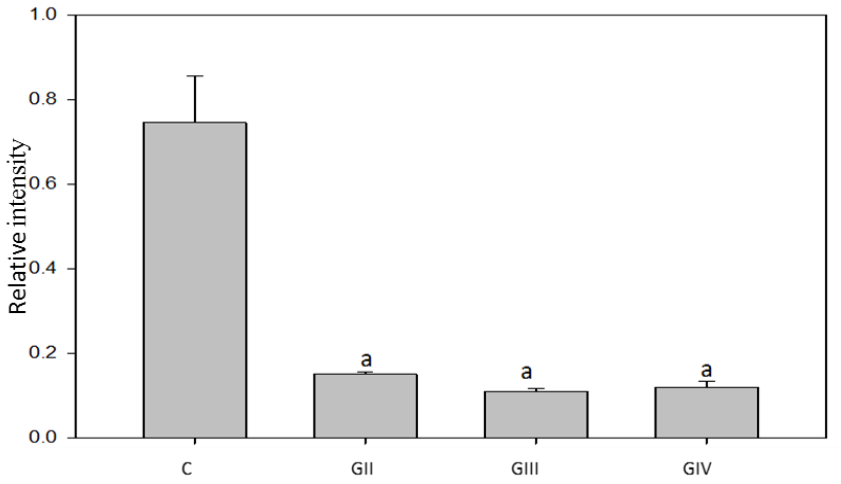


Fig. 26: Densitometric analysis of Western blot for SIRP Alpha in control, low (GII) and high grade (GIII and GIV) tissue samples. (n=45). Decrease in protein expression in low and high grade astrocytoma tissue samples was statistically significant compared with control brain tissue samples ($p< 0.05$).

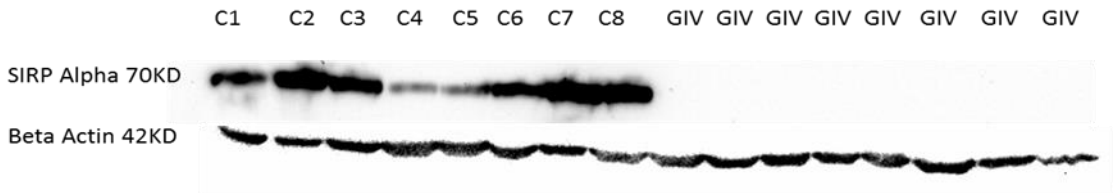


Fig. 27: Expression profile of SIRP Alpha in control brain and glioblastoma multiforme tissue lysates. C1, C2= Normal human brain tissue lysate, C3, C4, C5= temporal lobe epilepsy tissue lysate, C5, C6, C7= Frontal lobe epilepsy tissue lysate, GIV= glioblastoma multiforme tissue lysate.

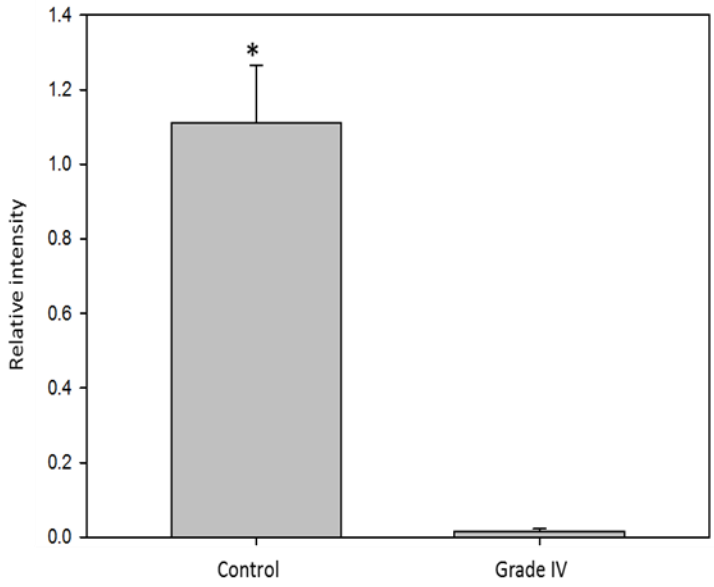


Fig. 28: Densitometric analysis of western blot image. Over expression in control brain tissue lysates were statistically significant as compared to glioblastoma multiforme tissue lysates.

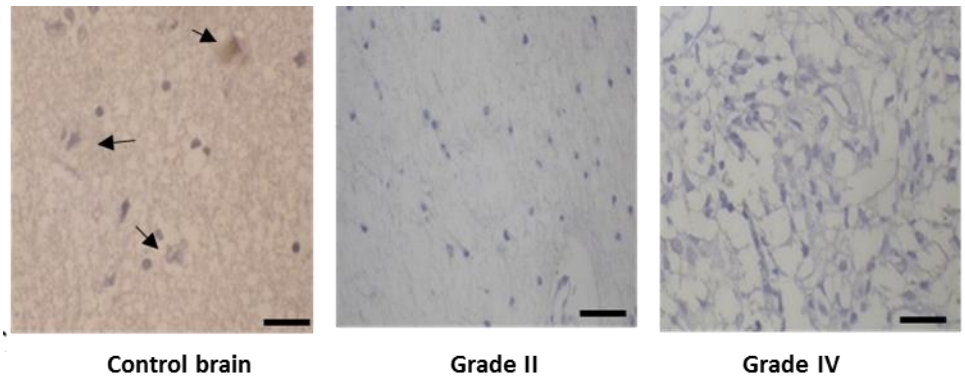
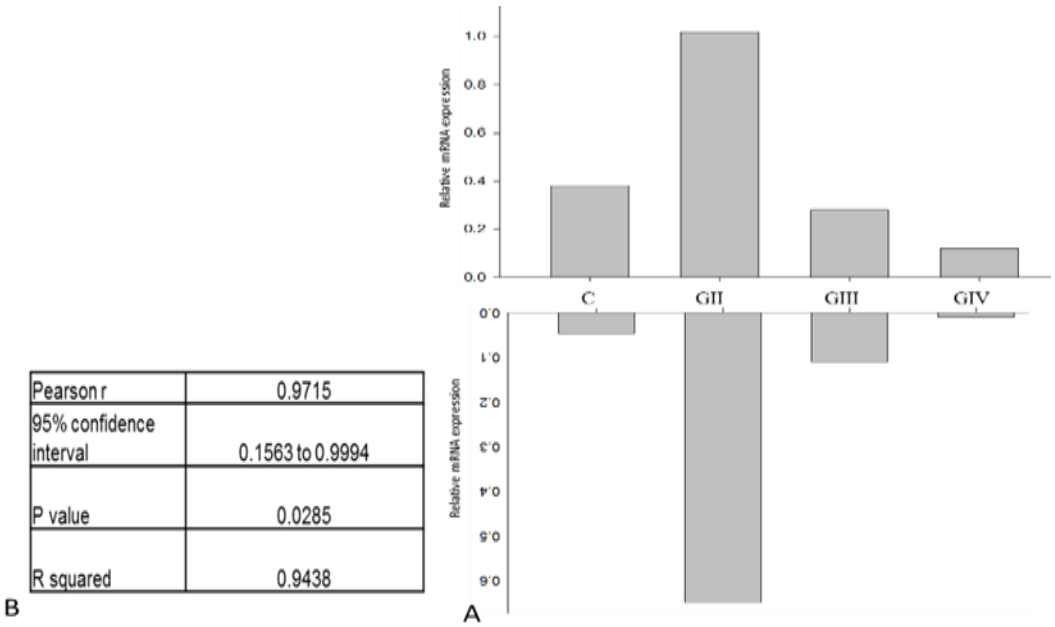


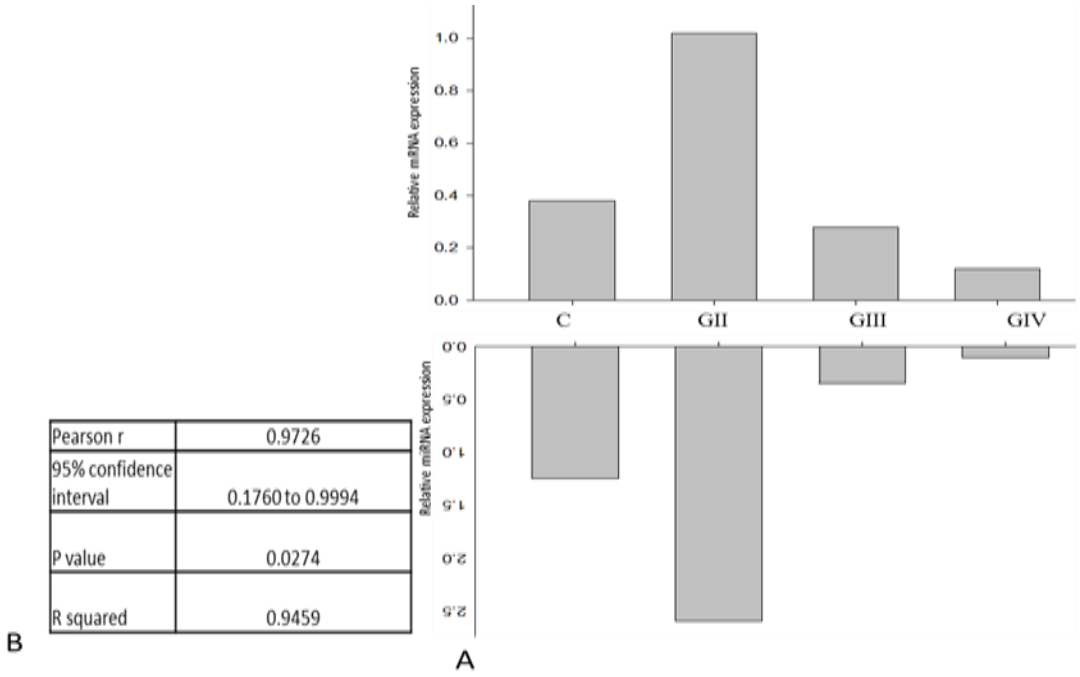
Fig 29: Immunohistochemistry staining for SIRP Alpha in control (C), low grade (GII) and high grade (GIV) tissue sections. Control brain sections were found to be positively stained while no significant staining was observed in low and high grade sections.



Pearson r	0.9715
95% confidence interval	0.1563 to 0.9994
P value	0.0285
R squared	0.9438

B

Fig.30: Correlation analysis of SIRP Alpha transcripts and Hsa-miR-520d-5p mature form by real time PCR in human astrocytoma tissue samples (A). Table (B) indicates expression patterns were observed with direct correlation. (p=0.0285, R= 0.94) in cohort of tissue samples.



B

Pearson r	0.9726
95% confidence interval	0.1760 to 0.9994
P value	0.0274
R squared	0.9459

Fig.31: Correlation analysis of SIRP Alpha transcripts and Hsa-miR-520d-3p mature form by real time PCR in human astrocytoma tissue samples (A). Table (B) indicates expression patterns were observed with direct correlation. (p=0.0274, R= 0.94) in cohort of tissue samples.

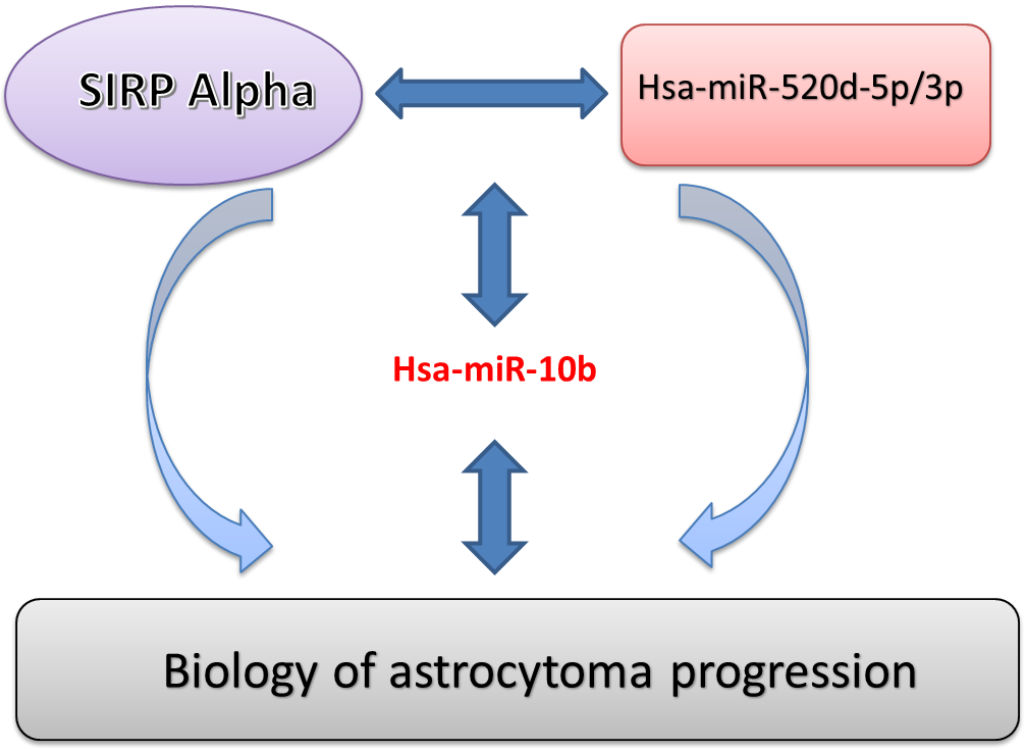


Fig. 32: Possible involvement of SRIP Alpha and miR-520d-5p, 520d-3p in biology astrocytoma progression.

Parameters	No of cases	SIRP Alpha protein expression		P Value
		Positive	Negative	
All cases	53			0.022
Age				
<=45	24	09	15	
>45	29	05	24	
Gender				<0.001
Male	26	07	19	
female	27	07	20	
WHO Grade				<0.001
II	11	00	11	
III	09	01	08	
IV	19	00	19	
Control	14	13	01	

Table 6: Correlation between SIRP Alpha protein expression with clinic-pathological parameters (n=53). Expression pattern was categorized into positive and negative on account of western blotting expression outputs.

3.6.4 Computational prediction of miRNAs targeting 3' and 5' UTR of SIRP Alpha transcript:

miRNAs were screened for putative targeting SIRP Alpha transcript. miRNAs collectively predicted by 3 or more programs were considered as strong putative candidates (Table 7). Further, selected candidate miRNAs were screened for SIRP Alpha 3'and 5' UTR binding efficiency. miR-520d-5p and miR-520d-3p were collectively predicted by 3 programs. 5' and 3' UTR score analysis strengthens (miR-520d-5p: 3' UTR score: -23.00, 5' UTR score:-23.00 and miR-520d-3p:3'UTR score: -29.3, 5'UTR score: -32.2) putative involvement of these miRNAs in SIRP Alpha post transcriptional regulation (Table 8).

Gene	miRNA	Target scan	miRnada	MirWalk	Miro	Total score
SIRP Alpha	miR-363	1	1	0	0	2
	hsa-miR-25	1	0	1	0	2
	hsa-miR-92a	1	0	0	0	3
	hsa-miR-92b	1	0	0	0	3
	hsa-miR-367	1	1	0	0	2
	hsa-miR-32	1	0	0	1	2
	hsa-miR-106a	1	1	0	1	3
	hsa-miR-106b	1	1	0	1	3
	hsa-miR-93	1	1	0	1	3
	hsa-miR-20a	1	1	0	1	3
	hsa-miR-20b	1	1	0	1	3
	hsa-miR-17	1	1	0	1	3
	hsa-miR-183	1	0	0	1	2
	hsa-miR-520d-5p	1	1	1	1	4
	hsa-miR-520d-3p	1	1	0	1	3
	hsa-miR-520b	0	1	1	1	3
	hsa-miR-520e	1	0	0	1	2
	hsa-miR-519d	1	1	0	1	3

Table 7: Computational prediction of miRNAs targeting SIRP Alpha transcripts. Putative hits were screened by four programs (Target scan, miRnada, miRWalk and miRo). miRNAs predicted by 3 or more programs were considered for further analysis.

miRNA	3' UTR score	5' UTR score
hsa-miR-20b-5p	-26.8	-22.2
hsa-miR-20b-3p	-29.7	-23.2
miR-520d-3p	-23.0	-23.0
miR-520d-5p	-29.3	-32.2
hsa-miR-519d-5p	-27.9	-27.0
hsa-miR-519d-3p	-25.7	-26.3

Table 8: targeting score of selected miRNAs to 3' and 5' UTR of SIRP Alpha mRNA.

Summary

3.7 Summary:

3.7.1 Expression profile and significance of CD200R:

CD200R and its ligand CD200R1 are reported to play a role in inflammatory reactions. CD200 is the only ligand reported to modulate the activity of CD200R1 and is conserved in human and mouse [16]. However, the role of CD200 in solid tumors is controversial in human squamous cell carcinoma [19], basal carcinoma [20] and melanoma [12]. Bi-directional role of immune activation in cancer is chiefly attributed to these conflicting evidences. Reduced CD200 expression in glioma cells have been reported to enhance microglia activation and tumor growth [17].

Here we have represented the expression profile of CD200R1 in human astrocytoma tissue samples. We found that initially, CD200R1 mRNA (Fig. 19) and protein (Fig. 20: A and B)expression is initially increased from control to low grade (GII) tumor tissue samples and decreased with further grade progression. Immunohistochemistry results further supplemented western blotting outcome. We find that CD200R1 was moderately expressed in control brain tissues. GII tissue sections were observed with more signal than control brain. There was no staining observed in GIV sections. The expression of CD200R1 expression was further found to be significantly associated with grade progression and not with age or sex of patients (Table 5). Recent report shows that CD200, ligand of CD200R1 systemically induce local inflammatory responses and shown to be an attractive target for breast cancer therapy. These investigations also show that absence of CD200R1 expression can lead to increased inflammatory cytokines in breast cancer [18].

Taken together we propose that as CD200R1 transcripts and proteins are significantly expressed in low grade tumor tissue samples and may have a role in progression of human astrocytoma from low to high grade. However, further investigations are necessary to know the molecular mechanisms of CD200R1 activity in astrocytoma progression.

3.7.2 Expression profile and significance of SIRP alpha:

In the present investigation, we have attempted to highlight expression profile of SIRP Alpha in human astrocytoma and control brain tissue samples. Our studies indicate that SIRP Alpha mRNA and protein is expressed in control brain tissues. Increased transcript expression in low grade (GII) was statistically significant compared to control and high grade tissue samples (Fig. 22). Further western blotting (Fig.25, Fig. 27) and densitometric (Fig. 26, Fig. 28) results depicts SIRP Alpha protein is expressed in control brain tissue. We did not find protein to follow the mRNA expression outcome in consequent grade progression. Of 11 GII tissue samples, none was positive for expression. Only one tissue among 9 GIII tissue was found positive and none among 19 GIV tissue was found with detectable levels of expression (Table 1). Immunohistochemistry profile supports western blotting results. SIRP Alpha protein expression was not detectable in low and high grade tumor tissue sections while control brain sections were moderately stained (Fig. 29). SIRP Alpha is reported to be under regulated in Dalton's lymphoma. Enhanced expression of SIRP Alpha and CD47 is reported to revert tumor associated macrophages to M1 state leading to tumor regression [37]. Cloning and overexpression experiments have shown SIRP Alpha expression leads to SHP2 mediated reduced cell migration and spreading in U87 malignant glioma cells [38].

We have computationally predicted miRNAs involved in regulation of SIRP Alpha expression. miR-520d-5p and miR-520d-3p were collectively predicted by 3 or more servers (Table 7). miRNA binds to 3' and 5'UTR of their targets and mediate post transcriptional inhibition of target mRNA

[39]. So, we have analyzed SIRP Alpha 3' and 5' UTR binding score of screened miRNAs and selected Hsa-miR-520d-5p (5'UTR score: -23kcal/mol 3'UTR score: -23kcal/mol) and Hsa-miR-520d-3p (5'UTR score: -32.2kcal/mol 3'UTR score: -29.3 kcal/mol) as they displayed perceivable binding energy with target mRNA (Table 7).

miR-520d-3p and miR-520d-5p mature form expression was validated by real time PCR. Initially, mature form of both miRNAs increased from control to GII progression, and decreased with subsequent high grade samples (Fig 23, 24). Increased expression in GII for miR-520d-5p was statistically significant as compared with control and high grade tissue samples (Fig. 24). Matured form levels of both miRNAs displayed similar pattern of expression as of SIRP Alpha mRNA. Further, we have studied the correlation between expression pattern of SIRP Alpha mRNA and predicted mature miRNAs. Hsa-miR-520d-5p was directly correlated with SIRP Alpha transcript expression (Fig 30 A and B) with $p=0.028$, $R= 0.97$. Hsa-miR-520d-3p mature form also directly correlated with SIRP Alpha mRNA expression (Fig 31 A and B) with $p=0.027$, $R= 0.94$. Based on computational targeting reports, mature form expression pattern by real time PCR and correlation studies, we presume that miR-520d-5p and miR-520d-3p may be involved in SIRP Alpha post transcriptional regulation.

miR-520d-3p is reported to be downregulated and associated with proliferation, invasion and migration in gastric cancer cases [40]. Studies in hepatocellular carcinoma reveals overexpression of miR-520d-5p is connected with diminished proliferation and invasiveness. miR-520d-5p is reported to mediate its effect through conversion of cancer cells to normal stem cells with maintained p53 expression [41]. Further, miR-520d expression in breast cancer has been associated with diminished proliferation and invasiveness. It is further noted to downregulate metastamiR miR-10b [42]. Recent investigation reveals miR-10b is upregulated in high grade astrocytoma tissue samples and correlated

with poor prognosis [43]. In astrocytoma, miR-10b is reported to mediate its effect through regulation of proapoptotic genes and cell cycle inhibitors [44] in turn inducing HOXD10 and MMP14 facilitating cell invasion [45]. Additional studies are needed to examine the association of miR-520d-5p and miR-10b in human astrocytoma malignancies. Recently, mice receiving injection of 520d-5p and T98 cells are reported to exist without malignant transformation, thus asserting the possible involvement of miR-520d-5p in glioblastoma progression [46]. Further studies warrant uncovering the underlying mechanisms of tumor suppressive activity of 520d-5p and miR-520d-3p in progression of malignant astrocytoma.

In conclusion, the interesting findings of our investigations are: SIRP Alpha transcripts are expressed significantly in GII tissue samples while SIRP Alpha protein was not detectable in low and high grade astrocytoma tissue samples. miR-520d-5p and miR-520d-5p expression pattern was directly correlated to SIRP Alpha mRNA expression, suggesting same miRNAs may be involved in SIRP Alpha post transcriptional regulation. As these miRNAs were significantly over expressed in GII tumor tissue samples as compared to control brain, GIII and GIV tissue samples, we further propose that miR-520d.-5p and miR-520d-3p could be used as diagnostic markers for low grade tumors. However, on account of present studies and available literature (Fig. 32), possible mechanisms of SIRP Alpha protein in biology of astrocytoma progression needs auxiliary investigations.

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Chapter IV

Prognostic significance of pDok2-Nck1 in astrocytoma progression.

Introduction

4.1 Introduction:

4.1.1 Biology of Dok2 and Nck1:

Downstream of tyrosine kinase (Dok2) belongs to a family of adapter protein reported to be responsive for the protein tyrosine kinase signaling. Dok family of proteins are known to localize on chromosome no 8. Dok family of protein has three domains: a N terminal homology domain, a phospho tyrosine bonding domain and a C terminal proline rich domain with few tyrosine residues. This C- terminal with sH2 and SH3 domain recruits the signaling intermediates on growth factor stimulation [1-4]. Epidermal growth factor receptor signaling is essentially viewed as an important player in shaping cancer microenvironment [1] and an important target for cancer therapy [5] . Dok1-3 are reported to modulate epidermal growth factor receptor [6, 7] as well as platelet derived growth factor receptor and associated signaling pathways [3, 8-10]. Knock out mutants with Dok-1/Dok-2 have been reported to be associated with leukemia genesis [11, 12]. Dok1 and Dok2 are related with an array of signaling pathways including TLR4[13], CD200R pathway[14] and by signaling lymphocyte activation molecule (SLAM) [15]. Dok1 and Dok2 have been preferentially known to be expressed on immune cells but predicted to have non immunological functions as well on account of its expression on cells of non-hematopoietic lineage [14, 16]. Studies have shown that Dok1 and Dok2 play an important role in TLR2 based signaling in astrocytes and microglia. This study also shows that activation of Dok1 and Dok2 results in cytokine production mediated by RasGAP dependent ERK and NF-$\varkappa$B activation. Dok1 and Dok2 are shown as novel targets for neuroinflammatory conditions [17]. In addition, Dok2 proteins have been connected with multiple biological processes including differentiation [18] and cellular motility [19, 20]. It has been reported that constitutive expression of Dok2 negatively regulates T cell development through interaction with RasGAP and Nck [21].

Dok2 expression has been reported to be associated with EGFR expression in lung adenocarcinoma and coupled with genomic loss of Dok2. This loss of Dok2 in genetically engineered mouse cooperates with EGFR to promote tumorigenesis [22]. In gastric cancer studies, Dok2 has been reported to express in normal gastric mucosa while difference in expression pattern was observed in gastric cancer cell lines. Further, these studies notes that Dok2 could be a useful marker to predict prognosis after curative resection [23]. Another study points that Dok2 was lost in 42% of gastric cancer cases. However, the concomitant molecular mechanisms in gastric cancer still remain at large [24]. In colorectal cancer studies, Dok2 has been reported to express in 66.7% of gastric cancer samples. It is further predicted as marker for poor prognosis[25]. Another independent study shows that Dok2 was lost on 36% of colorectal cancer [24]. However, the mechanisms behind these alterations are not known yet. Chronic myelomonocytic leukemia (CMML) studies shows that a mutation (L238P) in Dok2 is unable to inhibit ERK activation [26]. Studies in mouse erythroleukemia cells shows that Dok2 regulates the expression of Klf1 by binding to its promoter region and have a role in erythropoiesis. This study also shows that Dok2 is localized in cytoplasm and nucleus [27]. Epigenetic analysis of 45 ovarian cancer samples reveals that Dok2 gene underwent extensive methylation and its repression was found to increase carboplatin resistance by hampering apoptosis and anoikis[28]. We have reported that Dok2 protein and RNA are over expressed in low and high grade of astrocytoma tissue samples and its overexpression was correlated to poor prognosis. Real time PCR and western blotting results were supported by immunohistochemistry analysis. We further show that Dok2 expression was region specific with dominance of frontal and temporal lobes [29]. The details of Dok2 with reported target genes have been presented in Table 9.

Brain is protected from extracellular insults in several ways and one of them is by modulation of microglial activation. Dok2 have been shown to mitigate the production of inflammatory cytokines

induced by Aβ in turn activating the microglia. These effects were shown to be dependent of Cd200R modulation [30]. Further, Dok1 and Dok2 have been shown to react differentially to TLR stimulated signals in astrocytes and microglial cells. Dok1 and Dok2, on TLR activation, undergoes rapid phosphorylation and modulates the cytokine production through intermittent NF-κB and ERK activation.Dok1 and Dok2 represents the attractive targets to study microglial and astrocytic activation in inflammatory conditions [17].

Dok2 have been recently reported as an attractive marker for cancer progression. Its reported therapeutic value warrants further investigations. In addition, a better understanding of its downstream components in modulating immune responses could help to know its mechanism of action.

Little is known about expression profile and prognostic significance of Dok2 in astrocytoma context. Keeping in view the existing findings, here we have evaluated expression and prognostic significance of Dok2 in surgically resected astrocytoma tissue samples.

Gene	Cancer	Target gene	Reference
Dok2	Lung adenocarcinoma	Ras	24
	Gastric cancer	--	25,26
	Colorectal cancer	--	26,27
	chronic myelomonocytic leukemia (CMML)	ERK	28
	Erythroleukemia	Klf1	29
	Ovarian cancer	--	30
	Astrocytoma	--	31

Table 9: Association of Dok2 expression and downstream targets in cancers.

--: No clear evidences available.

Nck (non-catalytic region of tyrosine kinase) family constitutes two members, Nck1 and Nck2. These signaling proteins located in cytoplasm containing Src homology 2 and 3 domains [31]. Nck proteins are associated with receptor tyrosine kinases and regulate actin cytoskeleton dynamics including neuronal migration. It is also noted that Nck1 interacts with Dock, homologue of Dok in drosophila to commence the downstream signaling [32, 33]. Further, Nck1 is found to modulate endoplasmic reticulum stress associated signaling by regulating the activity of eukaryotic initiation factor 2 alpha [34]. Nck1 is also reported to promote cell migration by associating with dermcidin in hepatocellular carcinoma [35].

Materials and methods

4.2 Materials and Methods:

4.2.1 Human tissue samples: collection, processing and standard care followed:

In present study, surgically resected astrocytoma tissue samples were collected from Krishna Institute of Medical Sciences (KIMS) Secunderabad. Tissues were immediately snap frozen with liquid nitrogen and stored in -80^0 C for further analysis. Grade of astrocytoma samples were confirmed by criterion as enhanced cellularity, mitotic index, nuclear proliferation specified by World Health Organization. Initially, location of tumor was confirmed by Magnetic Resonance Imaging (MRI) and was broadly categorized in frontal, parietal, temporal and occipital lobes in brain. In case of tumors with overlap in two lobes, area with maximum tumor appearance was selected. For example, tumor with parieto-occipital appearance with maximum location in parietal lobe was regarded as parietal origin. Age/sex of subjects and follow up details were maintained at hospital registry. Temporal lobe epilepsy tissues were used as control. A part of tissue was used to isolate RNA for western blotting while another part was used for paraffin embedding which further used for immunohistochemistry studies. Tumor tissue was removed by near total, subtotal resection. Standard treatment includes radiotherapy and concomitant chemotherapy usually with temozolomide. Post-operative scan was monitored for every 3 and 6 months initially and then yearly to trace tumor recurrence. The studies are approved by the KIMS Foundation and Research Centre (KFRC), KIMS Ethical committee and was supported by informed consent form from the patients/ close relatives. Institutional ethical guidelines were followed. All the subjects were completely anonymized.

4.2.2 RNA isolation and real time PCR:

RNA was isolated from frozen samples by TRIzol method as described in manufacturer's instructions. RNA was quantified by NanoDrop spectrophotometer (Thermo Scientific). 5

micrograms of RNA was used for C-DNA synthesis by PrimeScript 1st strand C-DNA synthesis kit (TaKaRa, cat. No 6110A) following manufacturer's instructions. SYBR green (TaKaRa, cat.no. RR82LR) was used for real time PCR (Applied Biosystems, USA) analysis with equal concentration of C-DNA for each experiment. Primers were designed manually and GAPDH gene was used as internal control. (DOk2 Forward primer: TTTGGGCGGGACAAGGTAAC, Reverse primer: TCCAGGGCCAAGAAGATCTC, Tm: 60.5^0C, GAPDH forward primer: AAGGCTGGGGCTCATTTGCAG, Reverse primer: GCAGGAGGCATTGCTGATGATC, Tm: 60^0C). Samples were run in duplicates and for 35 cycles. Gene expression pattern was analyzed relative to internal control by $2-\Delta\Delta CT$ method [36].

4.2.3 Western blotting:

Approximately 100mg of tumor tissue was homogenized in RIPA buffer. Protease inhibitor cocktail (Sigma, cat no.: P8340) was added as per manual instructions. Protein concentration was estimated using Bradford method and 75 micrograms of protein was added with SDS sample buffer (0.125M Tris pH 6.8, 4% SDS, 20% glycerol, 0.2M DTT, 0.02% bromophenol blue). Protein extracts were then kept in boiling water bath for 5 minutes and used for loading in 10% SDS-PAGE. This was followed by transfer of protein from SDS gel to nitrocellulose membrane (Millipore) overnight in Towbin (0.048M Tris, 0.039M glycine, 20% methanol, 0.00375% SDS) buffer at 25 volts. 5% nonfat skimmed milk powder in TBST was used as blocking solution. Then, the blots were incubated with primary pDOk2 (Sc-13952, diluted to 1:500 in % skimmed milk solution) antibody. Blots were probed with alkaline phosphatase conjugated anti rabbit secondary antibody (Sigma, cat. No. A9919, Diluted to 1: 50000 in TBST) for 1hour at room temperature. Blots were developed by BCIP/NBT solution (Sigma, cat. No. B6404) in ALP buffer (100mM tris, 100mM NaCl, 50mM MgCl2) at room temperature.

4.2.4 Immunohistochemistry:

Immunohistochemical staining was performed using pDok2 primary antibody (1:200 dilution, Santa Cruz Biotechnology, USA). Paraffin embedded sections (5 microns) were prepared from surgically resected tissue using Leica microtome. Sections were dewaxed by heating on slide warmer (Yorco slide warmer) at 100^0C for 10 minutes. This step followed washes by xylene and series of alcohol for rehydration. Antigen retrieval was performed with citrate buffer (10mM sodium citrate, 0.05% of Twin20, pH 6.0) in microwave oven for 15 minutes. After cooling slides at room temperature, were incubated with primary pDok2 antibody. Further steps were followed as given in user manual of Invitrogen IHC detection kit (cat no: 87-9673).

4.2.5 Statistical analysis:

Statistical analysis was performed using SigmaPlot version 11.0. All results were represented as mean ± SD obtained from individual patients of each grade and were compared with control. The significant differences of the data were determined using one-way anova and log-rank test. Values of $P < 0.05$ were considered as statistically significant.

Results

4.3 Results:

4.3.1 Expression and significance of pDok2 expression in human astrocytoma:

4.3.2 Over expression of Dok2 transcripts in human astrocytoma:

Dok2 transcripts were found to be increased significantly in high grade astrocytoma tissue samples as compared with control brain tissues. GAPDH was used as internal control. The analysis was performed in total 47 tissue samples (07 control Brain, 10 GII, 11 GIII, 19 GIV tissue samples). Increased levels of transcripts in high grade astrocytoma (GIII and GIV) tissue samples was significant as compared with low grade (GII) and control brain tissue samples (Fig. 33).

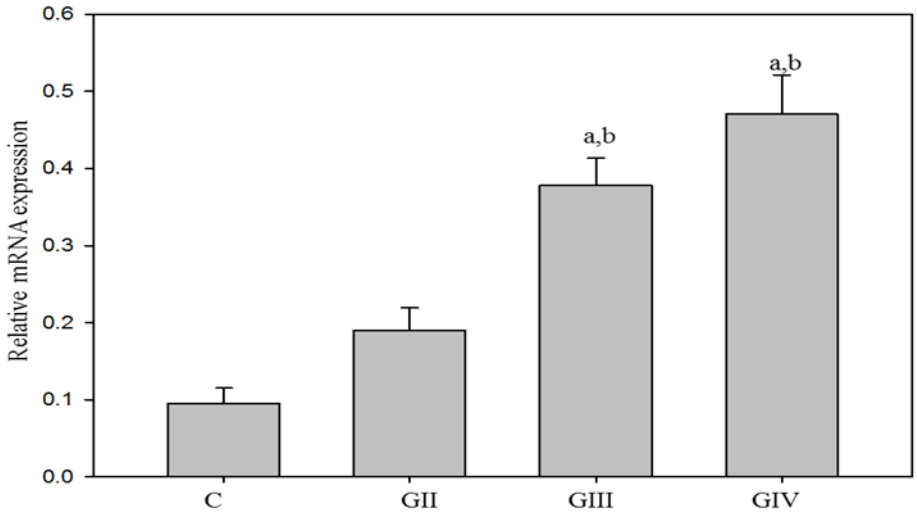


Fig.33 Real time PCR for Dok2 transcripts in control, grade II, grade III and grade IV tissue samples.

a= increased transcript expression in GIII and GIV tumor tissue was statistically significant as compared with control (a) brain tissues. ($p<0.05$)

b= increased transcript expression in GIII and GIV tumor tissue was statistically significant as compared with GII (b) tissues. ($p<0.05$)

C= control brain, GII= grade II, GIII= grade III, GIV= grade IV

4.3.3 pDok2 protein expression significantly altered in human astrocytoma tissue samples:

pDok2 protein levels were significantly expressed ($p<0.005$) in high grade astrocytoma (GIII, n= 11 and GIV, n=19) tissue samples compared with low grade (GII, n=10) and control (n=07) brain tissue samples. Of 7 control brain tissue samples, only 2 were found to express pDOk2 protein moderately (Fig 34). Densitometric analysis (Fig 35) reveals positive expression of protein in grade IV was statistically significant as compared with control brain ($p<0.001$) and grade II ($p=0.011$) tissue samples. Similarly, positive expression in GII and GIII was statistically significant as compared with control ($p=0.005$ and $p<0.001$ respectively). Further, western blotting outcomes were supported by immunohistochemistry experiments. High grade astrocytoma (GIV) sections were found to be positively stained with pDOk2 as compared with low grade (GII) and control tissue sections (Fig 36).

pDok2 expression was significantly correlated with progression of tumor grade ($p<0.001$). Age ($p=0.083$) and gender ($p=0.257$) of participants evaluated were not significantly associated with degree of pDok2 expression. Among the tissue samples used, (Control=7, GII=10, GIII-11, GIV=19), 16/19 GIV, 9/11 GIII, 3/10 GII samples were found to express pDok2 protein. Of 7 control brain samples, only 2 were detected with moderate pDOk2 expression. Overall, 25/30 high grade (GII+GIV) samples were reported to be positive with pDok2 protein (Table 9).

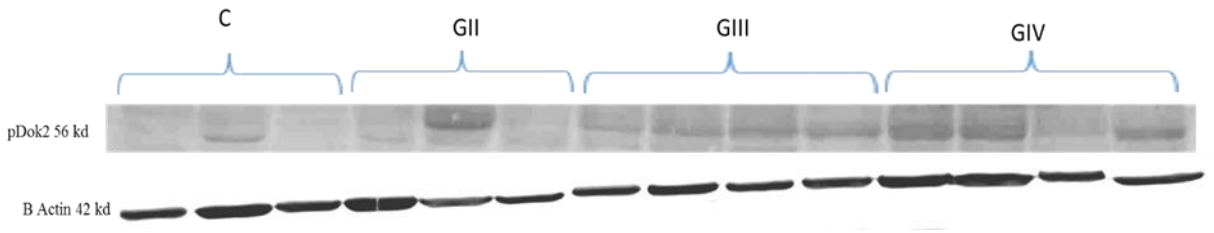


Fig. 34: Western blot for pDok2 protein expression in control, low grade (GII) and high grade (GIII and GIV) tissue lysates. Expression in high grade tissues was found to be significantly higher as compared to low grade and control brain tissues. Beta actin was used as internal control.

C= control brain, GII= grade II, GIII= grade III, GIV= grade IV

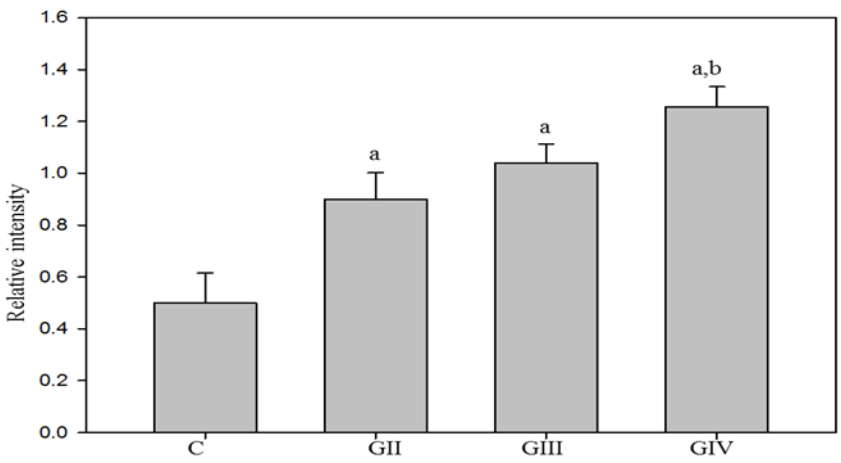


Fig.35: Densitometric analysis for pDok2 protein expression in control, grade II, grade III and grade IV tissue samples. Beta actin was used as internal control.

a= Increased pDok2 protein expression in GII, GIII and GIV tumor tissues were statistically significant as compared with control (a) brain tissue ($p<0.05$).

b= Increased expression in GIV tissue was statistically significant compared with control and GII (b) tumor tissue ($p<0.05$).

C= control brain, GII= grade II, GIII= grade III, GIV= grade IV

C GII GIV

Fig. 36: Immunohistochemistry analysis for pDok2 protein in control, low grade (GII) and high grade (GIII) astrocytoma tissue samples. Grade IV section was found with increased cellularity and cells with over expressing pDok2 as compared with control brain. Cell with positive staining are marked by arrow.

C=control brain, GII= grade two, GIV=grade IV

Parameters	No of cases	pDok2 Expression		P Value
		Positive	Negative	
All cases	47			0.083
Age				
<=45	18	11	7	
>45	29	19	10	
Gender				0.257
Male	30	19	11	
female	17	11	6	
WHO Grade				<0.001
II	10	3	7	
III	11	9	2	
IV	19	16	3	
Control	7	2	5	

Table 10: Association of pDok2 protein expression with clinico-pathological parameters evaluated in patients'.

4.3.4 Anatomic origin of tumor and follow up details:

Initially, tumor in brain was located by MRI imaging. In the present study, 17 females and 30 male subjects (gender ratio: 1.76) were included (Table 10). Tumor was found to be located for 13 cases in frontal lobe, 05 cases in parietal lobe, 17 cases in temporal lobe and 05 cases in occipital lobe (Table 11). Follow up of patients' was monitored for initial interval of 3 and 6 months and then per year.

Patient sr. no	Age/sex	Tumor type	Location of tumor	Follow up time (in months)
1	51/M	Grade III	Frontal	25
2	48/M	Control	Temporal	31
3	38/M	GBM	Temporal	35
4	55/F	Grade II	Parietal	20
5	46/M	GBM	Frontal	32
6	44/M	Control	Temporal	38
7	29/F	Grade III	Temporal	23
8	58/M	Grade II	Temporal	24
9	42/M	GBM	Frontal	31
10	49/M	Grade III	Parietal	29
11	52/M	GBM	Temporal	15
12	34/F	GBM	Frontal	40
13	37/M	Grade II	Temporal	41
14	49/F	GBM	Frontal	41
15	50/M	Grade II	Temporal	N.A.
16	56/F	GBM	Temporal	12
17	25/M	GBM	Temporal	14
18	61/M	Grade III	Frontal	20
19	41/M	Grade III	Temporal	19
20	62/F	GBM	Frontal	29
21	44/M	Grade III	Parietal	N.A.
22	53/F	GBM	Frontal	24
23	56/M	Control	Temporal	29
24	35/F	GBM	Temporal	15
25	57/M	Control	Temporal	26
26	31/F	Grade III	Frontal	24
27	51/M	GBM	Temporal	13
28	28/F	Grade II	Occipital	16
29	30/M	Grade II	Temporal	24
30	55/F	GBM	Frontal	29
31	46/M	Control	Temporal	N.A.
32	15/F	Grade II	Temporal	9
33	47/M	GBM	Temporal	7
34	52/M	GBM	Temporal	9
35	41/F	Grade II	Parietal	24
36	55/M	Grade III	Frontal	10
37	58/M	Grade II	Parietal	N.A.
38	15/M	GBM	Occipital	11
39	59/F	Grade III	Occipital	15
40	60/M	GBM	Occipital	12
41	61/F	Grade II	Occipital	17
42	65/M	GBM	Frontal	N.A.
43	46/M	Grade III	Frontal	28
44	35/F	Grade III	Temporal	24
45	52/M	Control	Temporal	38
46	39/F	GBM	Temporal	N.A.
47	36/M	Control	Temporal	35

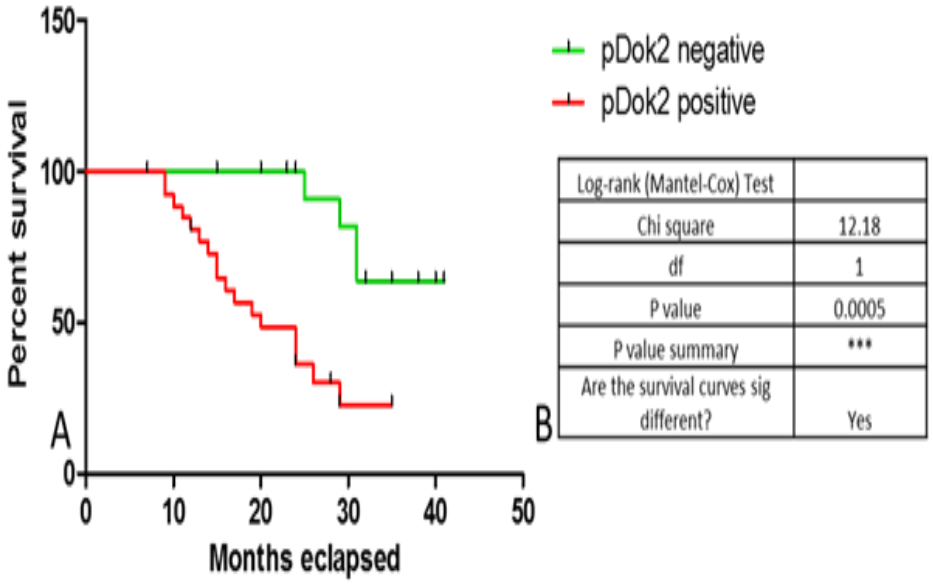
Table 11: Anatomic origin of tumor and follow up details.

Lobe / Grade	Frontal	Parietal	Temporal	Occipital
Grade II	01	03	04	02
Grade III	04	02	04	01
Grade IV	08	00	09	02
Total	13	05	17	05

Table 12: Distribution of tumor locations on the basis of anatomic origin in brain.

4.3.5 pDok2 expression and patients' prognosis:

To evaluate association of pDok2 protein expression with patients' prognosis, expression pattern was categorized into two groups with the help of pathologists: pDok2 positive and pDok2 negative. This categorization was based on account of degree of pDOk2 protein expression and confirmed by two independent observations. Control and few GII tissues were used as reference on account of observed low expression of pDok2 protein. Log-Rank test was used to evaluate the effect of pDok2 expression on prognosis of astrocytoma patients'. Of 47 cases, follow up details were not available for 5 patients'. As shown in Fig.5, total 42 cases were studied. Positive expression of pDok2 was displayed in 26 cases while negative expression in 16 cases. Significant difference was observed among pDok2 positive and pDok2 negative group (Fig. 37, B) with p= 0.0005, chi square=12.8 and 95% CI 0.083 to 0.49. Patients' with positive pDok2 expression were found to have median survival age of 20 months.



Log-rank (Mantel-Cox) Test	
Chi square	12.18
df	1
P value	0.0005
P value summary	***
Are the survival curves sig different?	Yes

Fig. 37: Kaplan-Meier survival curve (A) with log-rank test (B) for patients with positive (red line, n=26) and negative (green line, n= 16) pDok2 expression. Median survival of 20 months was reported with patients with high pDok2 expression (95% CI 0.083 to 0.49).

4.3.6 Expression and significance of Nck1 expression in human astrocytoma:

4.3.7 Nck1 transcripts over expressed in human astrocytoma tissue samples:

Real time PCR expression reveals that Nck1 transcripts were increased in GIV compared with GIII and control brain tissue samples. High grade astrocytoma tissue (GIII and GIV) samples showed elevated transcripts compared with low grade astrocytoma and control brain tissue samples. GAPDH gene was used as internal control and fold change in expression was analyzed using 2 (Delta Delta C (T)) method.

Nck1 transcripts were found to be upregulated from control to high grade progression. Increase in transcript expression in high grade tissues were statistically significant ($p<0.005$) compared with control and low grade tissues (Fig. 38).

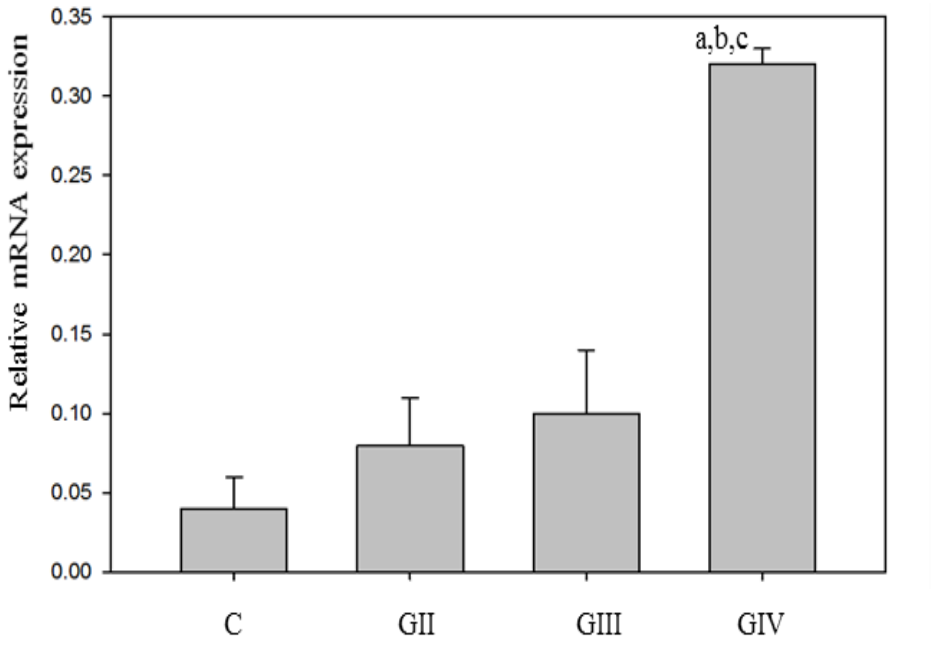


Fig 38: Real time PCR expression analysis of Nck1 in control, low grade (GII) and high grade (GIII, GIV) tumor tissue samples. Relative mRNA expression in GIV tissues was significantly increased compared with control (a), GII (b) and GIII (c) ($p<0.005$). GAPDH was used as internal control.

C= control brain tissue, GII= grade II astrocytoma tissue, GIII= grade III astrocytoma tissue, GIV= grade IV astrocytoma tissue.

4.3.8 Expression of Nck1 protein was significantly altered in 43 human astrocytoma tissue samples:

Western blotting results were consistent with real time PCR analysis. Nck1 protein was highly expressed in GIV tumor samples. Overall, densitometric analysis reveals Nck1 was overexpressed in high grade tumor tissue (GIII and GIV) compared to low grade (GII) and control brain tissue. Beta actin was used as internal control (Fig.38).

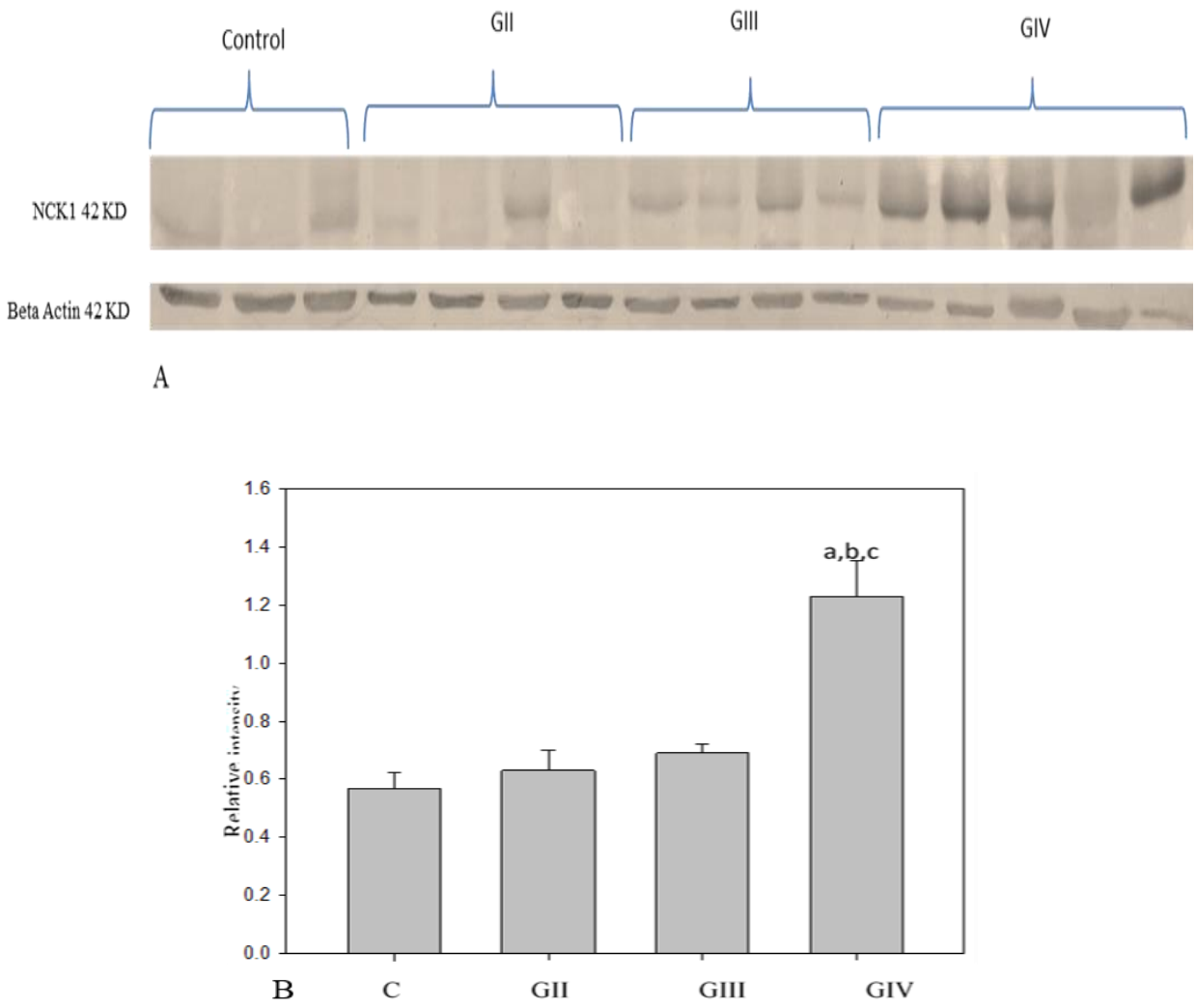


Fig 39: Western blotting (A) Nck1 (1:1500 dilution) and densitometric analysis (B) of Nck1 in control, low grade (GII) and high grade (GIII, GIV) tumor tissue samples. Protein expression in GIV tissues was significantly increased compared with control (a), GII (b) and GIII (c). Internal Nck1 expression were normalized with Beta actin (internal control) C= control brain tissue, GII= grade II astrocytoma tissue, GIII= grade III astrocytoma tissue, GIV= grade IV astrocytoma tissue.

Of total 43 samples we studied, 26 cancer tissues were positive for expression Nck1. Out of total subjects under study, 28 were males and 15 were females. 14 out of 17 GIV, 9 of 11 GIII and 3 of 8 GII tumor tissues showed Nck1 overexpression. We have used total 07 control brain samples, of which only 02 samples were positive Nck1expression while 05 samples showed no expression. 23/28 high grade tissues were positive for Nck1expression (P<0.005) (Table: 13)

Parameters	No of cases	Nck1 protein Expression		P Value
		Negative Nck1 expression	Positive Nck1 expression	
All cases	43			<0.001
Age				
<=45	18	7	11	
>45	25	8	17	
Gender				0.156
Male	28	10	18	
female	15	5	10	
WHO Grade				<0.001
II	8	5	3	
III	11	2	9	
IV	17	3	14	
Control	7	5	2	

Table 13: Correlation of Nck1 protein expression with pathological indicators in astrocytoma clinical tissue samples.

Patient sr. no	Age/sex	Tumor type	Location of tumor	Follow up time (in months)
1	46/M	GBM	Temporal	25
2	47/M	GII	Frontal	30
3	25/M	C	Temporal	55
4	32/F	GII	Frontal	24
5	41/M	GIII	Temporal	24
6	18/F	C	Temporal	24
7	55/M	GBM	Temporal	30
8	32/F	GIII	Frontal	14
9	49/M	GII	Parietal	35
10	27/M	C	Temporal	29
11	59/M	GBM	Temporal	9
12	35/M	GBM	Parietal	35
12	60/F	C	Temporal	58
14	51/M	GIII	Frontal	N.A.
15	42/M	GBM	Temporal	N.A.
16	56/F	C	Temporal	9
17	47/M	GII	Temporal	24
18	50/M	GBM	Temporal	12
19	55/M	GIII	Occipital	14
20	61/F	GBM	Temporal	20
21	58/M	GIII	Temporal	19
22	45/M	GII	Temporal	29
23	46/F	GBM	Frontal	24
24	48/M	GIII	Frontal	24
25	52/M	GIII	Occipital	29
26	51/F	GBM	Occipital	15
27	09/M	GBM	Frontal	26
28	21/F	GBM	Temporal	24
29	38/F	GIII	Frontal	13
30	57/M	GBM	Temporal	16
31	40/M	GIII	Temporal	28
32	47/M	C	Temporal	12
33	25/F	GII	Temporal	8
34	48/M	GBM	Temporal	9
35	18/F	GII	Occipital	35
36	51/M	GBM	Temporal	9
37	23/F	GII	Temporal	24
38	50/M	GBM	Frontal	10
39	34/F	GIII	Parietal	7
40	49/M	GBM	Temporal	N.A.
41	32/F	C	Temporal	15
42	48/M	GBM	Parietal	12
43	17/M	GIII	Occipital	11

C: control, GII: grade II, GIII: grade III, GBM: glioblastoma multiformae, N.A.: data not available

Table 14: Anatomic origin of tumor and follow-up information of patients'.

Lobe / Grade	Frontal	Parietal	Temporal	Occipital
Grade II	02	01	04	00
Grade III	04	01	03	04
Grade IV	03	03	10	01
Total	09	05	17	05

Table 15: Anatomic origin of astrocytoma subtypes.

Case	n	Nck1 positive	Nck1 negative	*p*
Normal Brain	7	2	5	<0.001
Cancer tissues	36	26	10	<0.001
Low grade (GII)	8	3	5	<0.001
High grade (GIII + GIV)	28	23	5	<0.001

Table 16: Status of Nck1 protein expression in control and astrocytoma tissue samples.

4.3.9 Immunohistochemistry analysis:

Normal brain tissue, low grade tissue (GII) and high grade (GIII, GIV) tumor tissue were used for immunohistochemistry studies. Nck1 protein was detected in high grade (GIII and GIV) tissue samples significantly than low grade (GII) and control brain tissue (Fig. 40). Overall, IHC results supports western blotting in terms of Nck1 protein up regulation in GIV astrocytoma tissue samples compared with control brain tissue.

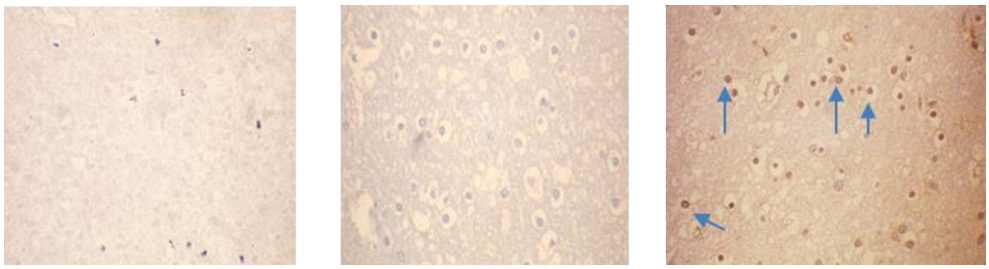


Fig 40: Immunohistochemical staining of Nck1 (1:250 dilution) in control, low grade (GII) and high grade (GIV) tumor tissue samples and analyzed microscopically. Cells with positive staining in Grade IV sections are marked with an arrow.

4.3.10 Correlation of Nck1 expression with clinicopathological parameters:

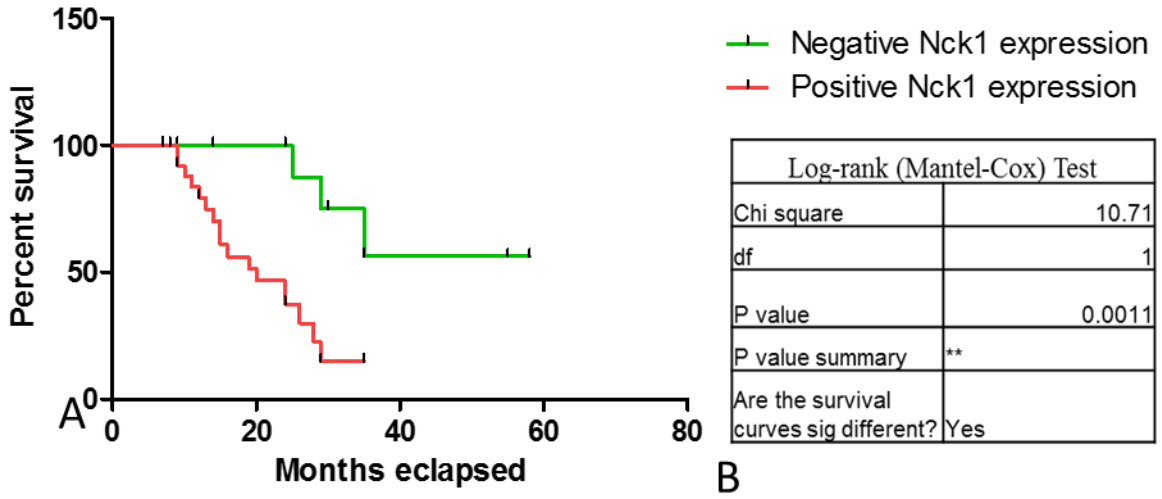
Nck1 protein expression was assessed in control brain, diffuse astrocytoma, anaplastic astrocytoma and glioblastoma multiformae tumor tissue samples. Expression pattern was categorized with the help of pathologists as positive or negative depending on the signal of protein expression. Correlation of protein expression was evaluated with other clinicopathological parameters such as age, sex and tumor grade levels. Nck1 expression was correlated with grade progression ($p<0.001$), age ($p<0.001$) and was not correlated with gender of patients' ($p= 0.156$) (Table 13).

4.3.11 Anatomic origin of tumor and standard care followed:

Location of tumor was determined from MRI imaging. Excision of tumor was done either near total or subtotal ways. There were 28 males and 15 females in our study group (gender ratio: 1.2). Tumor location was found 09 in frontal, 05 in parietal, 17 in temporal and 05 in occipital area. (Table 14, 15) Follow up was monitored every 3, 6 months and then yearly (Table 13). In cases diagnosed with grade IV astrocytoma (glioblastoma multiformae), after operative resection (either near total or subtotal), radiation and chemotherapy cycles (12 months). Scanning was done every 3 months and monitored for recurrence.

4.3.12 Nck1 expression levels may be correlated to clinical prognosis of astrocytoma:

Nck1 expression was correlated to patients' prognosis in 41 cases. Follow up details were not available for 2 cases. To estimate the details, Nck1 positive and Nck1 negative groups were maintained as described previously. Over expression was correlated with poor survival outcome in patients. Long-rank test evaluated that patients' with high Nck1 expression had shorter survival time ($\chi 2$ = 10.7, P value = 0.0011) (Fig. 41). Median survival time of 20 months was reported with Nck1 positive group.



Log-rank (Mantel-Cox) Test	
Chi square	10.71
df	1
P value	0.0011
P value summary	**
Are the survival curves sig different?	Yes

Fig. 41: Kaplan-Meier survival curve (A) with log-rank test (B) for patients with positive (red line, n=27) and negative (green line, n= 14) Nck1 expression. Median survival of 20 months was reported with patients with high Nck1 expression (95% CI 0.0861 to 0.540).

Summary

4.4 Summary:

4.4.1 pDok2:

In present investigations, we demonstrate for the first time expression profile and prognostic importance of pDok2 expression in human astrocytoma tissue samples. Real time PCR (Fig.33) signifies pDok2 transcripts are significantly expressed in high grade (GIII+GIV) tissue samples as compared with low grade (GII) and control brain tissues ($p<0.005$). Western blotting (Fig. 34 and 35) and immunohistochemistry (Fig.36) analysis signifies over expression of pDok2 protein in surgically resected tissues as compared with control brain tissues. High grade (GIII+GIV) tissue samples were found considerably express pDok2 protein as compared with control and low grade (GII) tissue samples ($p<0.001$). Clinico-pathological analysis reveals degree of pDok2 expression was meaningfully correlated with grade progression ($p<0.001$) and not with age ($p=0.083$) or sex ($p=0.257$) of patients' (Table 9). We have further seen the prognostic significance of pDok2 expression. Patients with pDok2 expression were found with significantly lower survival than with under-expressed pDok2 ($p= 0.0005$) (Fig 37 A and B, Table 10).

In agreement with our results, previous studies show that pDok2 is upregulated in moderately differentiated colorectal adenocarcinoma and can serve as biomarker for poor prognosis in patients after curative restriction [13]. Studies in gastric cancer reports Dok2 to be upregulated in 50% gastric cancer samples and could be used as marker for prediction of prognosis in clinical studies [37]. Overexpressed Dok2 have been shown to promote angiopoietin mediated cell migration by influencing actin cytoskeleton rearrangement [15]. Angiopoietin1/ Angiopoietin2 balance was shown to have prognostic value in astrocytoma patients [14]. However, the association of Dok2 and angiopoietin signaling is elusive in astrocytoma progression. Dok family of protein appears to be

functionally conserved among the lineage. Dock, a homologue of Dok family of proteins have been shown to play a role in axonal guidance in drosophila [16].

Mice with knocked out Dok1 (homologue of Dok2) and Dok2 developed myeloproliferative disorder resembling myeloid leukemia in human [17]. Studies in lung cancer [18] have reported tumor suppressive function of Dok2. Loss of Dok2 in ovarian cancer [19] has been shown to induce chemotherapy resistance in response to treatment by decreasing apoptosis levels. Mutational analysis of Dok2 in leukemia [20] reveals no detectable somatic mutations. This suggests another mechanism as deletion of locus may be prevalent. Further studies are needed to ascertain the multiple components of Dok2 and its effector mechanisms.

Further, we have seen anatomic origin of tumor locations in brain. In total, 13 tumors were located in frontal lobe, 05 in parietal lobe, 17 in temporal lobe and 05 in occipital lobe (table: 11). We found predominantly frontal and temporal lobe as anatomic site for glioblastoma multiforme. Of total 19 glioblastoma multiforme cases we studied, in 08 cases tumor was located at frontal lobe, in 09 cases at temporal lobe and in 02 cases at occipital lobe. Overall we observed non uniform anatomic distribution of tumor location. It highlights possible role of transcription or developmental factors involved at particular locations present findings are in agreement with previous studies regarding dominated occurrence at frontal and temporal lobes [21].

In conclusion, present study shows that pDok2 was found to express significantly in 84% of GIV astrocytoma tissue samples. In total, 83% of high grade astrocytoma (GIII+GIV) and 30% of low grade (GII) tissue samples were detected with pDok2 expression. Clinicopathological and survival studies underline the use of pDok2 protein as marker for prediction of prognosis in astrocytoma patients.

4.4.2 Nck1:

In present study, we report for the first time that Nck1 mRNA and protein is significantly expressed in astrocytoma tissue samples as compared with control brain tissue. Here, high grade (GIII and GIV) tissue samples found to overexpress Nck1 significantly than low grade (GII) tissue samples. Control brain tissue samples were found with weak expression. Western blotting results were supplemented by immunohistochemistry (IHC) analysis. IHC expression pattern underlines predominant staining in GIV tissues (Fig 40). Nck1 expression enhanced with malignancy grade 3/8 (Grade II), 9/11 (Grade III), 14/17 (Grade IV) in astrocytoma tissue samples ($p< 0.005$). Therefore, Nck1 overexpression may contribute to progression and aggressiveness of astrocytomas. Overall, we found 37% of grade II, 81% of GIII and 82% of GIV tissue samples were positive for Nck1 expression.

We have looked into anatomic origin of tumor (Table 14, 15). Of all cases we studied, 09 were found to be located in frontal lobe, 05 in parietal lobe, 17 in temporal lobe and 05 in occipital lobe. Of 17 grade IV cases reported, 10 were located in temporal lobe, 03 in parietal lobe, 03 in frontal lobe and 01 in occipital lobe. Overall, we found non uniform distribution of tumors with predominance of frontal and temporal lobes in our cohort reflecting possible involvement of functional or developmental factors involved. Previous report supports our findings of prominent frontal and temporal dominance [38].

Nck1 is well characterized family of adaptor protein with SH2/SH3 domains. Sos protein has been shown to be one of the interacting partner of Nck [39] resulting in Ras activation [40] Nck1 is shown to play a role in axonal guidance and have been overexpressed in murine colon carcinoma cell lines CT51, CT26, and in human breast cancer cell lines MCF7, T47D. Nck2, a homologue of Nck1 is reported to promote melanoma cell migration and invasion by modulating actin cytoskeleton [41]. Nck1 expression is reported prevent tunicamysin induced CHOP expression and

apoptosis in Hep G2 and MCF-7 cell lines, thus playing a role in counteracting deleterious effect of ER stress in cancer cell survival [41]. Expression profile and its prognostic significance of Nck1 in astrocytoma pathology is not assessed. Considering the available information on biology of Nck1 expression, we evaluated expression profile and prognostic significance of Nck1 in astrocytoma progression.

We further correlated Nck1 expression with overall survival of astrocytoma patients (Table 13). Log-rank test showed that patients with positive Nck1 expression displayed overall survival of 20 months. The difference between two groups was statistically significant with $p= 0.001$, Chi square = 10.7 (Fig 41). Clinicopathological analysis (Table 13) reveals Nck1 expression was statistically correlated with age of patients' and grade progression ($p< 0.005$). Cumulatively, 82% of high grade astrocytoma tissue were with positive expression of Nck1 (Table 16). Recent report reveals phospho- elF2 beta recruits Nck1 to elF2 leading to elF2 dephosphorization. This events are part of mTORC1 signaling in HEK293E cells. Further, Nck1/Elf2 complex is also reported as novel target for metastatic breast cancer [42]. However, this pattern of Nck1 signaling remains to be explored in astrocytoma progression. Previous reports [41, 43, 44] have supported our observations that Nck1 expression could be an attractive target for therapeutic intervention.

Taken together the present findings, we propose Nck1 as a valuable biomarker for astrocytoma prognosis with dominated appearance at frontal and temporal lobe. A better understanding of its downstream targets and mechanisms undelaying non-uniform anatomic distribution could yield modified therapeutic value.

4.5 References:

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Chapter V

In-vitro evaluation of cell arrest induced by nitidine chloride.

Introduction

5.1 Introduction:

Nitidine is a phytochemical alkaloid derived from the roots of Zanthoxylum nitidum. Nitidine chloride (NC) is a chloride derivative of nitidine (Fig. 50 A) and possesses anti-inflammatory, analgesic, and antifungal bioactivities [1]. NC has been reported to inhibit hepatocellular carcinoma and renal cancer proliferation [2, 3]. Studies on breast cancer showed that NC inhibits metastasis by suppressing c-SRC/focal adhesion kinase (FAK) pathway [4]. Recently, NC has been reported to inhibit malignant glioblastoma cells by targeting PIK3/AKT/mTOR axis [5].

The present study aimed at investigating the anti-glioma proprieties of NC on C6 and U87 glioma cell lines. The study established the migration and proliferation inhibitory effects of NC in vitro. It has also been shown that NC elevates caspase 3 and caspase 7 dependent cell death. Taken together, the present findings shed light on anti-glioma activity of NC and the potential need to understand its activity.

Materials and methods:

5.2 Materials and methods:

5.2.1 Cell lines and reagents:

The C6 and U87 cell lines were procured from the National Center for Cell Sciences (NCCS) Pune, India and were cultured in DMEM medium (HIMEDIA, Cat no. AL007A-500ML) with 10% FBS (GIFCO, USA), antibiotic, antimycotic solution (GIFCO, USA, Cat no. 10270). Antibodies beta-actin (cat. No. 4970), cleaved caspase 3 (cat no. 9915), and Gsk3 beta (cat no.: 9315) were purchased from Cell Signaling Technology, USA. Secondary HRP antibodies and NC (cat. No. SML0610-5MG) were procured from Sigma-Aldrich, USA.

5.2.2 MTT assay for cell viability:

C6 (1×10^4 cells/ well) and U87 (1×10^4 cells/ well) cells were seeded in 96 well plate and incubated at 37°C at a concentration of 5% CO_2. After overnight incubation, cells were treated with different concentrations of NC (2-14 µmol) for 24 h. After treatment, each well was added with 20 µl of 5 mg/ml of MTT dissolved in autoclaved miliQ and incubated for 3 h in dark. MTT was subsequently removed, and 50 µl of DMSO was added to each well. The plates were shaken at room temperature for 20 seconds, and the absorbance was measured at 570 nm by ELISA reader (Bio-Rad, USA). All experiments were repeated three times.

5.2.3 Colony formation assay:

C6 cells were made in a single cell suspension. Around 500 cells/well were seeded in a six-well plate and incubated for 24 h at 37°C with 5% CO2. The cells were then treated with three different concentrations of NC (4, 8, and 12 µmol) for 24 h. Media in the six-well plate was replaced with fresh one and cultured for 8 days. The media was again refreshed after 3 days. The cells were then fixed

with 4% paraformaldehyde (PFA) for 10 minutes at room temperature. Further staining with 2% crystal violet was performed. Visible colonies were counted. Images were taken with Olympus digital camera (Olympus, Japan).

5.2.4 Cell cycle analysis by flow-cytometry:

Flow cytometry was used to analyze the distribution of cells upon NC treatment. C6 cells were seeded at 1×10^5 cells/ well density in the six-well plate for 24 h. The cells were then treated with NC for 24 h. Total cells were collected by centrifugation (Eppendorf centrifuge- 5418R) at 1500 rpm for 5 minutes and washed with phosphate buffer saline (PBS). The cells were then fixed with 70% ethanol for 30 minutes at room temperature. Cells were collected by centrifugation at 2000 rpm for 5 minutes and washed with PBS. Further staining was done with propodeum iodide (50 µg /ml) and RNase A (1mg/ml) solution. Cell cycle analysis was performed with BD LSRFortessa flow cytometer (BD Biosciences, USA). The data was analyzed using FlowJo (Oregon) analysis platform.

5.2.5 Wound healing assay:

C6 cells (1×10^5 cells) were seeded in a six-well plate. After the cells reached confluency, a wound was marked with a blunt tip. Dislodged cells were removed, and fresh 2 ml medium supplemented with 10% serum was added. Percentage inhibition of wound healing was assessed in treated experimental sets as compared to control.

5.2.6 Western blot:

C6 wells were seeded in 20 mm Petri plate with 3 ml of DMEM media supplemented with 10% of serum. After the cells reached 70% of confluency, they were treated with NC for 24 h. Control cells were treated with vehicle control (DMSO). After 24 h, the cells were washed with PBS and lysed in RIPA buffer supplemented with protease and phosphate inhibitor cocktail (Sigma, cat no.: P8340).

The cell lysate was then used for protein estimation by Bradford's reagent (Sigma, cat. No. B6916). An equal amount of protein (50 μg) was mixed with 6x loading dye. It was boiled for 3 minutes and used for loading with 12% SDS polyacrylamide gel. Proteins in the gel were transferred to nitrocellulose membrane (Millipore) with Towbin buffer overnight at 25 V for 12 h. Cleaved caspase 3, Gsk3 Beta, pDok2, Nck1 primary antibodies were dissolved in 5% skim milk powder mixed with TBST (1:500 dilution for each antibody). Blots were blocked with 5% skim milk solution for 1 h followed by primary antibody incubation overnight at 4°C. After subsequent washing with TBS and TBST, the blots were probed with secondary antibody (1:10000 dilution) for 1 h at room temperature. The blots were then developed with VersaDoc (Bio-Rad, USA) molecular imager.

5.2.7 Statistical analysis:

The results were expressed as mean +SD. SigmaPlot 11.0 was used for statistical analysis. Student's t test was used to analyze the other data. Error bars represented standard error of three independent experiments. $P<0.005$ was considered as statistically significant.

Results

5.3 Results:

5.3.1 NC inhibits glioma cell proliferation:

In the present study, we have investigated the anti proliferative effect of NC on C6 and U87 cell lines using MTT assay. NC displayed dose-dependent inhibition of cell growth in U87 (Fig. 42) and C6 (Fig. 43) cell lines. In U87 cell lines, the IC50 value appeared to be around 10 µmol while in C6 cell line, it was around 8 µmol (Fig. 44). Furthermore, we have seen the effect of NC on colony formation in C6 cell line (Fig. 45). The effective concentrations of MTT assay were selected. Here, it was clear that NC displayed IC50 value at 8 µmol. These results were consistent with the initial MTT assay.

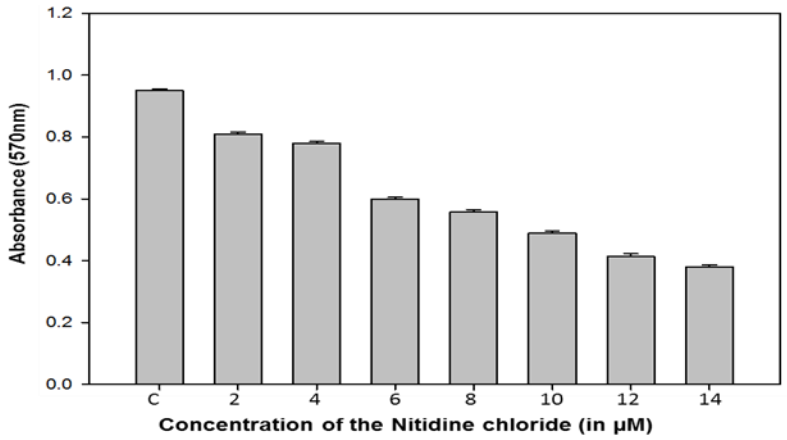

Fig 42: Effect of nitidine chloride (NC) on U87 cell line (2-14 micromoles-24 hrs). NC was found to induce cell death in U87 cells in a concentration-dependent way. The degree of induced cell death at 8, 10, 12, and 14 micromoles was statistically significant as compared with control ($p<0.005$).

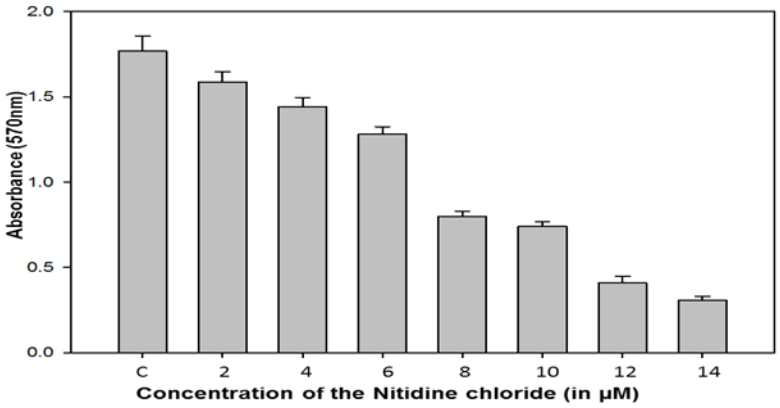

Fig 43: Effect of nitidine chloride (NC) on C6 cell line (2-14 micromoles-24 hrs). NC was found to induce cell death in C6 cells in a concentration-dependent way. The degree of cell death induced at 8, 10, 12, and 14 micromoles was statistically significant as compared with control ($p<0.005$).

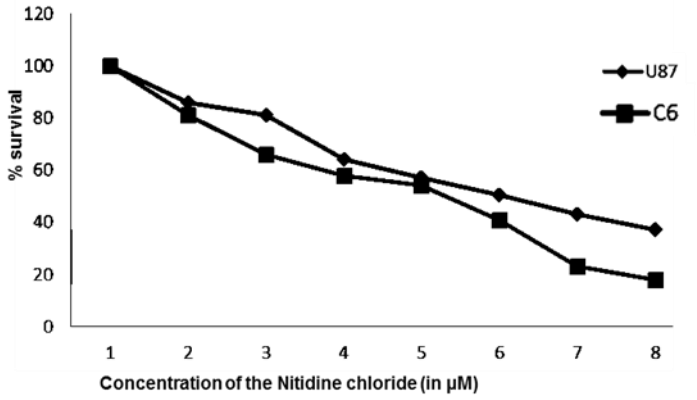

Fig 44: Effect of nitidine chloride (NC) on U87 and C6 cell line (24 hrs). C6 and U87 cells were observed with IC50 value of around 8 micromoles.

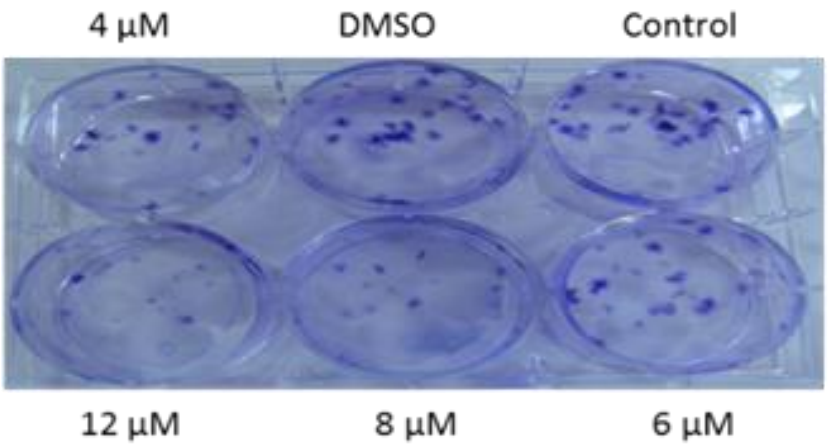

Fig 45: Effect of nitidine chloride (NC) on C6 cells proliferation. NC was shown to arrest colony formation at effective concentration of 8 micromoles.

5.3.2 NC induced G2/M cell cycle arrest in C6 glioma cell line:

The effect of NC on cell cycle arrest was assessed in C6 glioma cell line by flow cytometry. C6 cells were treated with four varied concentrations (4, 6, 8, and 12 µmol) of NC. The control cells were treated with empty vehicle. It is clear from Fig. 46 (A, B, C, D, E) that NC-induced cell arrests in G2/M phase of cell cycle in a dose-dependent way that was accompanied by a decrease in G0/G1 phase (Fig.46-F). In C6 cells, the percentage of cells in G2/M phase was increased from 28% to 52%. In cells treated with 8 µmol of NC, 50% of cells were found to be arrested in G2/M phase of cell cycle. The results suggested that NC arrests C6 cells in G2/M phase of cell cycle.

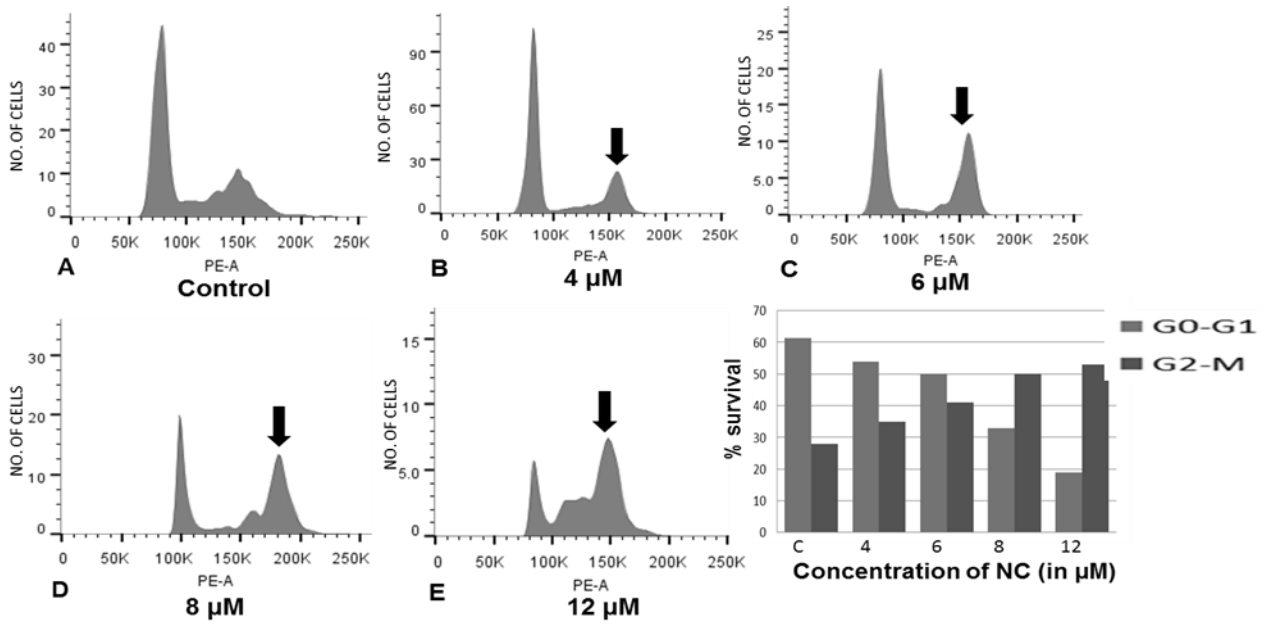

Fig. 46: Cell cycle analysis of nitidine chloride (NC) in C6 cell line treated with empty vehicle (A), 4 (B), 6 (C), 8 (D), and 12 micromoles (E). Cell cycle arrest was observed in G2/M phase of cell cycle (F).

5.3.3 NC inhibits wound healing of C6 cells:

It is clear from Fig. 47 that NC treatment resulted in inhibition of wound healing in C6 cells in a dose-dependent manner (A, B, C, D, and E). Cells in control panel were treated with empty vehicle. The percentage increase in diameter was calculated as compared with the control. Cell treated with 6 and 8 µmol of NC showed 30% and 35% increase in diameter, respectively, as compared to control. Cells treated with 4 µmol were found to increase 30% in diameter. Cells treated with 12 µmol were found to display nearly 70% increase in diameter as compared to control (F).

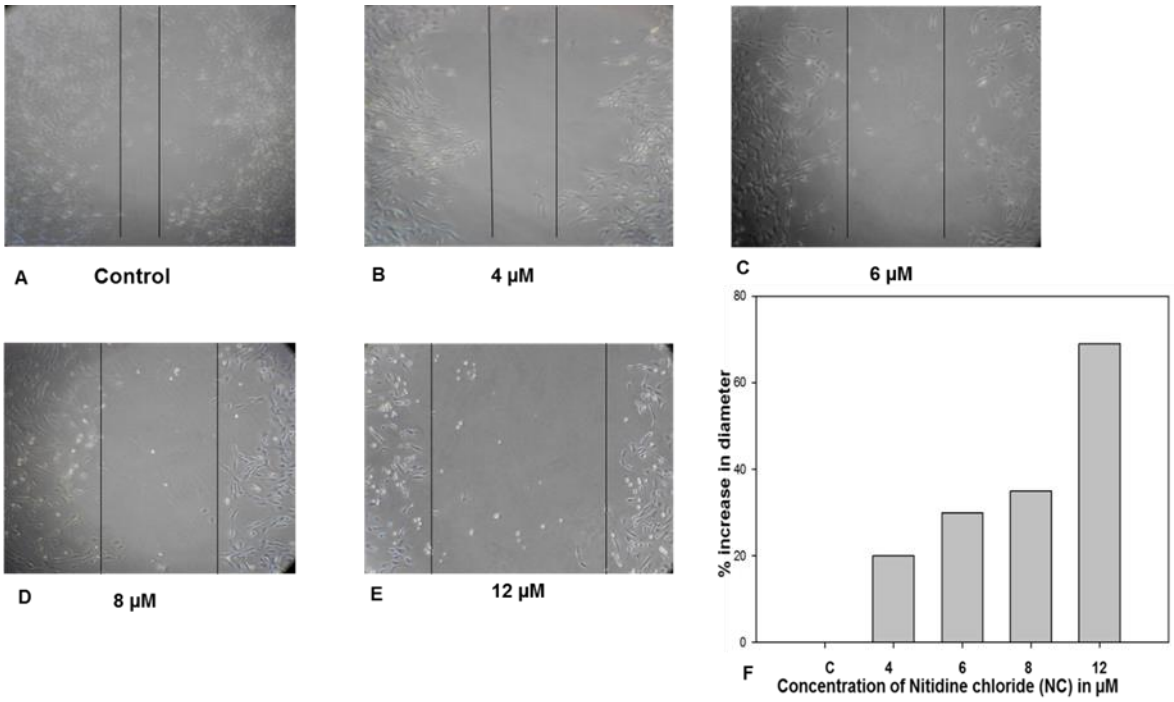

Fig. 47: Wound healing assay for C6 cell line treated with empty vehicle (A), 4 micromoles (B), 6 micromoles (C), 8 micromoles (D), and 12 micromoles (E). Diameter of wound was calculated as percentage increase compared with control. It is evident that the diameter of wound gradually increased with effective nitidine chloride (NC) concentration (F).

5.3.4 NC induces apoptosis through alteration of caspase and Gsk3 beta:

Western blot experiments (Fig. 48 A) demonstrated that NC-induced cell death in caspase 3 dependent ways. C6 cells were treated with 4, 8 and 12 µg of NC. Cell death was found to increase with dose and was statistically significant ($p<0.05$) in 8 and 12 µg treatment module as compared to control (Fig. 49 C). Conversely, we found that pGsk3 expression diminished with progressive NC treatment. Decrease in pGsk3 beta in C6 cells treated with 12 µmol of NC was statistically significant ($p<0.05$) as compared to control for 4 and 8 µmol (Fig.49 D). Further, we also found that cleaved PARP and cleaved caspase 7 were overexpressed on NC treatment. Increased expression of cleaved PARP and caspase 7 in C6 cells treated with 12 µmol of NC was found to be statistically significant as compared to empty vehicle

-treated cells (Fig 49 A and B). Furthermore, we also have assessed the effect of NC on pDok2 and Nck1 protein expression, which has been recently reported as a possible therapeutic target for human malignant glioma. It was found that NC inhibited pDok2 protein expression in U87 malignant human glioblastoma cell line. In addition, the decrease in pDok2(Fig.49: E) and Nck1 (Fig.49: F) protein expression in U87 cells treated with 12 µmol of NC was statistically significant as compared to empty vehicle treated control U87 cells.

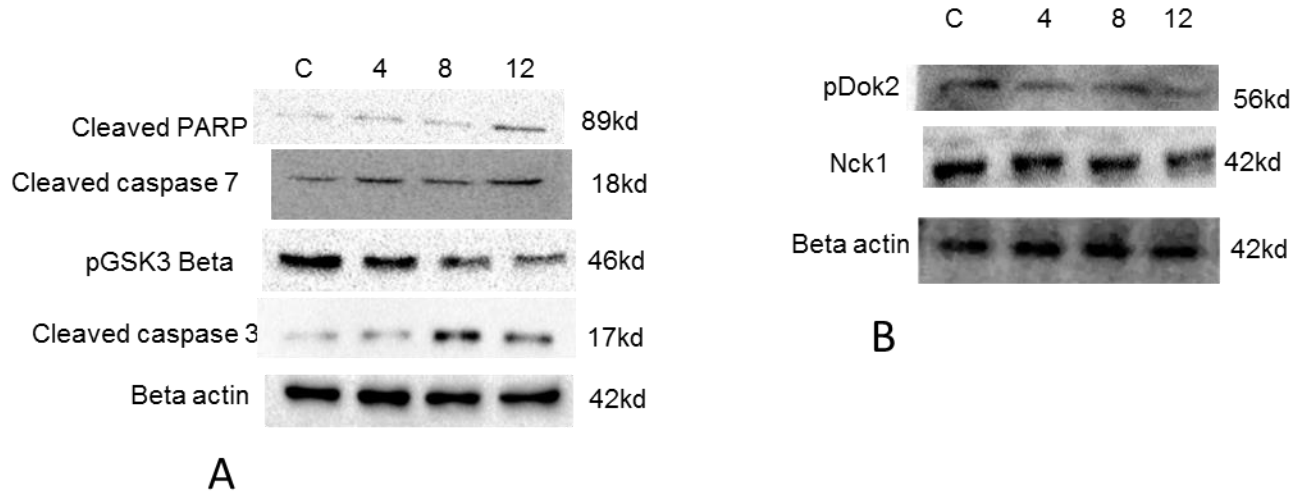

Fig. 48: Western blot analysis (A) for nitidine chloride (NC)-mediated induction of cleaved PARP, cleaved caspase 7, pGSK3 beta, and cleaved caspase 3 in C6 cell line. (B) It represents NC-mediated down regulation of pDok2, Nck1 expression in U87 human malignant glioma cell line.

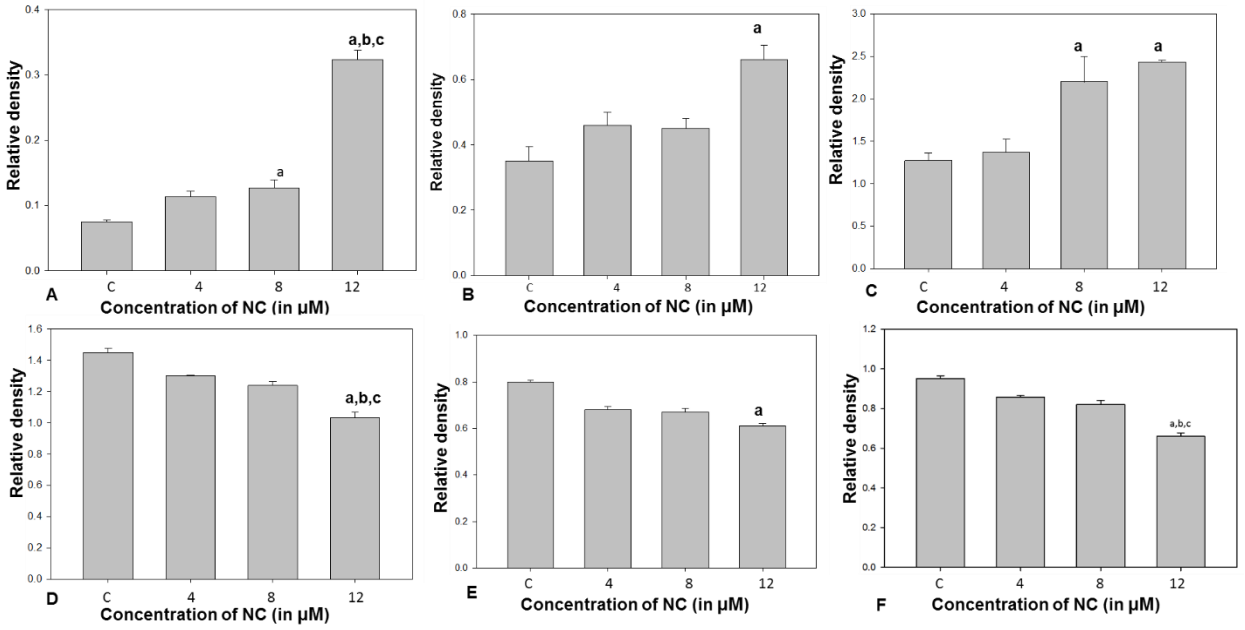

Fig.49: Densitometric analysis for cleaved PARP (A), cleaved caspase 7 (B), caspase 3 (C), pGSK3 beta (D) in nitidine chloride (NC) treated C6 cell line. pDok2 (E) and Nck1 (F) was found to be down regulated in NC treated U87 cells. Cleaved caspase 3 expression was found to increase as a function of NC treatment while Gsk3 Beta expression was decreased with NC treatment. Increased expression of caspase 3 for 8 and 12 micromoles was statistically significant ($p<0.05$) as compared with control. Decreased expression of Gsk3 Beta for 12 micromoles was statistically significant ($p<0.05$) as compared with control, 4, 6 and 8 micromoles.

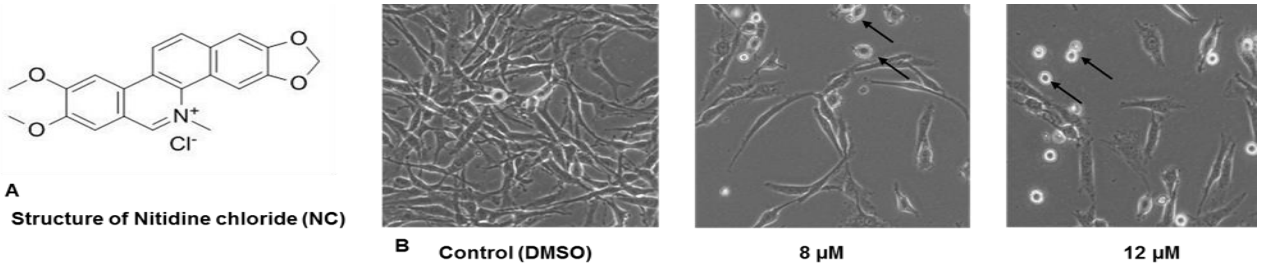

Fig. 50: Structure of nitidine chloride (NC) (A) and morphological appearance (B) of C6 cells on NC treatment. Cells with altered morphology have been marked by an arrow.

Summary

5.4 Summary:

Traditional herbs are being widely used to extract bioactive compounds including alkaloids, terpenoids, and flavonoids. Recently, researchers have focused on its possible use for anticancer treatment. Accumulating literature has amply justified the anti-cancer activities of NC on various malignancies including renal cancer, gastric adenocarcinoma, and lung adenocarcinoma [6-8]. NC has been shown to exert mTOR-dependent cell arrest activity in U87 human glioma cells [5]. However, the activity of NC has not been reported in rat glioma cells as they are most widely used in preclinical investigations. Our results shed light on anti-cancer activities of NC on rat glioma cells (C6). Further, Dok2 has been reviewed as a potential target in colorectal and gastric carcinoma [9, 10]. Recently, we have shown that pDok2 protein significantly expressed itself in human glioblastoma multiforme and is correlated with poor prognosis in clinical cases [11]. We have also reported that NC inhibits Dok2 and Nck1 protein expression in a dose dependent-way and can be viewed as a therapeutic target.

In this study, we showed that NC inhibits glioma cells proliferation in a dose-dependent way. The IC50 value of 8 μmol was found to be consistent as validated by MTT (Fig. 42, 43 and 44) and clonogenic assays (Fig. 45). NC was also found to inhibit wound healing at effective concentrations of 8 and 12 μmol. Cells treated with 12 μmol of NC were found to show 70% increase in diameter as compared to control (Fig. 47). The present results have been supported by the existing literature study [7]. We have accessed the cell death on NC treatment with propidium iodide (PI) staining by flow cytometry. It was evident from the results that NC treatment arrests the C6 cells in G2/M phase of cell cycle. Cells treated with NC (4, 6, 8 and 12 μmol) showed a progressive decrease in the cell population in G0-G1 phase of cell cycle while there was an increase in the percentage of cells in G2/M phase of the cell cycle (Fig. 46). The present results have been supported by similar studies on breast cancer cell lines [12]. Here, NC was reported to arrest MCF-7 and MDA-MB-231 cells in G2/M phase

of the cell cycle. This arrest was accompanied by a decreased number of cells in S phase of cell cycle. Western blotting results demonstrated that NC treatment induced caspase 3 dependent cell death at previously verified concentrations. The upregulation of cleaved caspase 7 in C6 cells at 12 μmol concentration was found to be statistically significant as compared to C6 cells treated with vehicle, 4 and 8 μmol of NC, respectively ($p<0.001$). It was found that NC inhibits Gsk3 Beta protein expression. The degree of Gsk3 Beta suppression in cells treated with 12 μmol of NC was statistically significant as compared to control and cells treated with 4 and 8 μmol of NC. Recently, NC has been shown to inhibit akt, a component of Gsk3 beta cascade, and has been an active candidate for possible therapeutic intervention [5]. NC was also found to exert an inhibitory effect on cleaved PARP and caspase 7 proteins, which are reported to be deregulated in glioma biology [11, 13-15]. We observed the cleaved PARP protein expression that progressively increased from control to NC-treated (4, 8, and 12 μmol) cells. The degree of upregulation in glioma cells treated with 12 μmol of NC was statistically significant as compared to control and glioma cells treated with 4 and 8 μmol of NC ($p<0.001$). Similarly, we observed cleaved caspase 3 protein expression that was significantly upregulated in C6 glioma cells treated with 8 and 12 μmol as compared to control.

In summary, our findings imply that NC alters cell morphology (Fig. 50), exerts arrest of cell cycle and colony formation in C6 cells, and is supplemented with its inhibitory effect on wound healing activity. Further investigations are necessary to ascertain the downstream targets to uncover its therapeutic potential.

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compromises stem cell phenotype and survival of glioblastoma-initiating cells. *Cell death and differentiation* 2014, 21(2):258-269.

Summary:

Chapter II:

Astrocytomas are highly reported neoplasms of central nervous system with middle aged groups' beings more prone. Patients with glioblastoma multiformae were found with lower median age (37.5) than western population (62). Headache and seizures were observed more frequently in high grade glioma as compared to low grade glial tumors. Overall tumor anatomic origin at frontal lobe in 34% of cases, at parietal lobe in 11.8% of cases, at temporal lobe in 39.8% of cases and at occipital lobe in 14.1% of cases. Tumor located at frontal lobe was found to have better prognostic value than temporal, parietal and occipital lobes. Glioblastoma with oligodendroglial component was found to have better prognostic value and respond well to chemo and radiotherapy. We found median age of survival (in months) 24 for pilocytic astrocytoma, 25 for diffuse astrocytoma, 19 for anaplastic astrocytoma and 12 for glioblastoma multiformae. 55% of GBMOs were found to be positive for p53.

Chapter III:

CD200R1 and SIRP Alpha mRNA expression was found to be initially increased in GII tissue samples as compared with control brain tissue and decreased with grade progression. CD200R1 protein expression was found to follow the mRNA expression pattern and supported by IHC studies. SIRP Alpha protein levels were more in control than compared with low and high grade tumor samples, these results were supported by immunohistochemistry outcome. Computational prediction reveals miR-520d-3p and miR-520d-5p as strong putative hits with possible role in regulation of SIRP Alpha post translational expression. Matured form levels of both miRNAs (miR-520d-5p and miR-520d-3p) displayed similar pattern of expression as of SIRP Alpha mRNA.

Chapter IV:

Semi quantitative PCR results shows pDok2 and Nck1 transcripts are overexpressed in high grades (III and IV) as compared with low grade glioma and control brain tissue samples. Western blotting and IHC results shows pDok2 and Nck1 protein was found to be significantly overexpressed in high grade astrocytoma tissue samples. In total, 83% of high grade astrocytoma (GIII+GIV) and 30% of low grade (GII) tissue samples were detected positive with pDok2 expression. Clinico-pathological and survival studies underline the use of pDok2 as marker for prediction of prognosis in astrocytoma patients' with overall survival of 20 months. 82 % of high grade glioma confirms upregulation of Nck1. Log-rank test showed that patients with positive Nck1 expression displayed overall survival of 20 months. Frontal and temporal lobe was found to be the dominant anatomic origin for both pDok2 and Nck1 protein expression.

Chapter V:

NC induces cell death in glioma cell lines (C6 and U87). NC inhibits glioma cell (C6) proliferation and migration in dose dependent way. FACS results suggest that NC arrests cell growth at the G2/M phase. These results are supported by similar studies in breast cancer cell lines. Further, NC was found to inhibit pDok2 and Nck1 which are reported to be over expressed in high grade astrocytoma tissues.

Manuscripts Published:

1. Deshpande RP, Chandra Sekhar YB, Panigrahi M, Babu PP. Region specific Dok2 overexpression associates with poor prognosis in human astrocytoma. **Molecular Neurobiology**. 2016. doi: 10.1007/s12035-016-0324-2. **ISSN: 1559-1182.**

2. Deshpande RP, Chandra Sekhar YB, Panigrahi M, Babu PP. SIRP Alpha protein downregulates in human astrocytoma: presumptive involvement of Hsa-miR-520d-5p and Hsa-miR-520d-3p. **Molecular Neurobiology.** 2016. pp 1–8. doi:10.1007/s12035-016-0302-8. **ISSN: 1559-1182.**

3. Deshpande RP, Babu D, Panigrahi M, Chandra Sekhar YB, Prakash Babu P. Brain tumors incidences and a retrospective clinical analysis from a tertiary hospital in India. J **Neurooncol.** 2016; 129(2):383-7. **ISSN: 1573-7373.**

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4. Deshpande RP, Babu PP. pdok2, caspase 3 dependent glioma cell growth arrest by nitidine chloride.

Journal Name: Pharmacological Reports **Manuscript No.:** 971-4873-2

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5. Deshpande RP, Babu PP. Understanding Dok2: from tumor biology to neubiological functions.

Journal Name: Annals of Neurosciences **Manuscript No.:** 971-4873-2

6. Deshpande RP, Chandra Sekhar YB, Panigrahi M, Babu PP. Prognostic significance of anatomic origin and evaluation of survival statistics of astrocytoma patients' in a cohort with clinico-pathological assessment of glioblastoma with oligodendroglial component

Journal Name: BMC Cancer **Manuscript No.:** BCAN-D-17-00069

7. Deshpande RP, Chandra Sekhar YB, Panigrahi M, Babu PP. Expression profile and significance of hsa-miR-519d-3p and hsa-miR-519d-5p in human astrocytoma progression

Journal Name: Neurological Research **Manuscript No.:** NER5006

Manuscripts Finalized:

8. Deshpande RP, Chandra Sekhar YB, Panigrahi M, Babu PP. Over expression of Nck1 protein in human astrocytoma is associated with poor prognosis with prominent anatomic origin at frontal and temporal lobe

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LETTER TO THE EDITOR

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

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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.

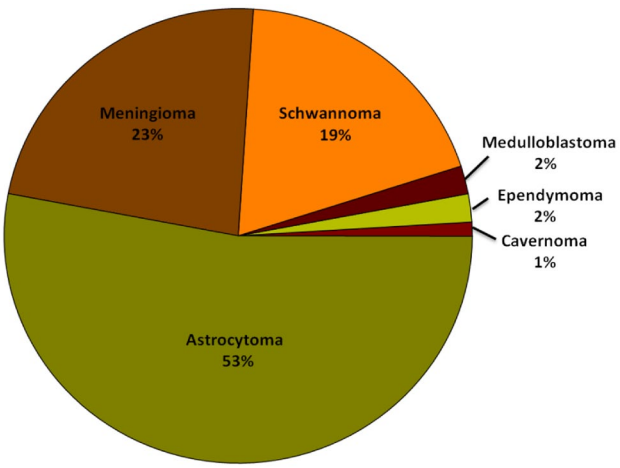

Fig. 1 Statistical distribution of brain tumors among different age groups in analyzed cohort

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

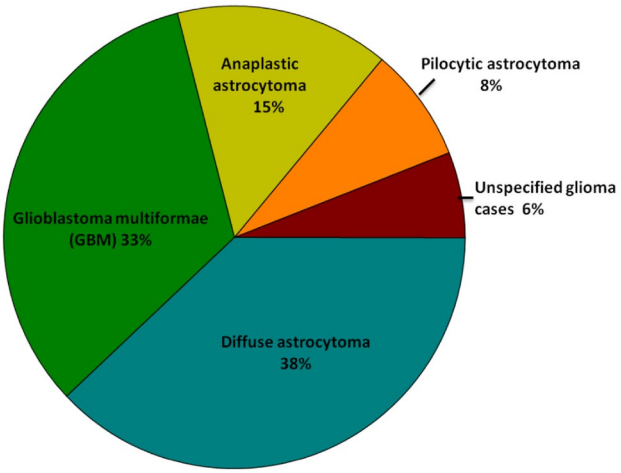

Fig. 2 Statistical distribution of astrocytoma subtypes among different age groups in analyzed cohort

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

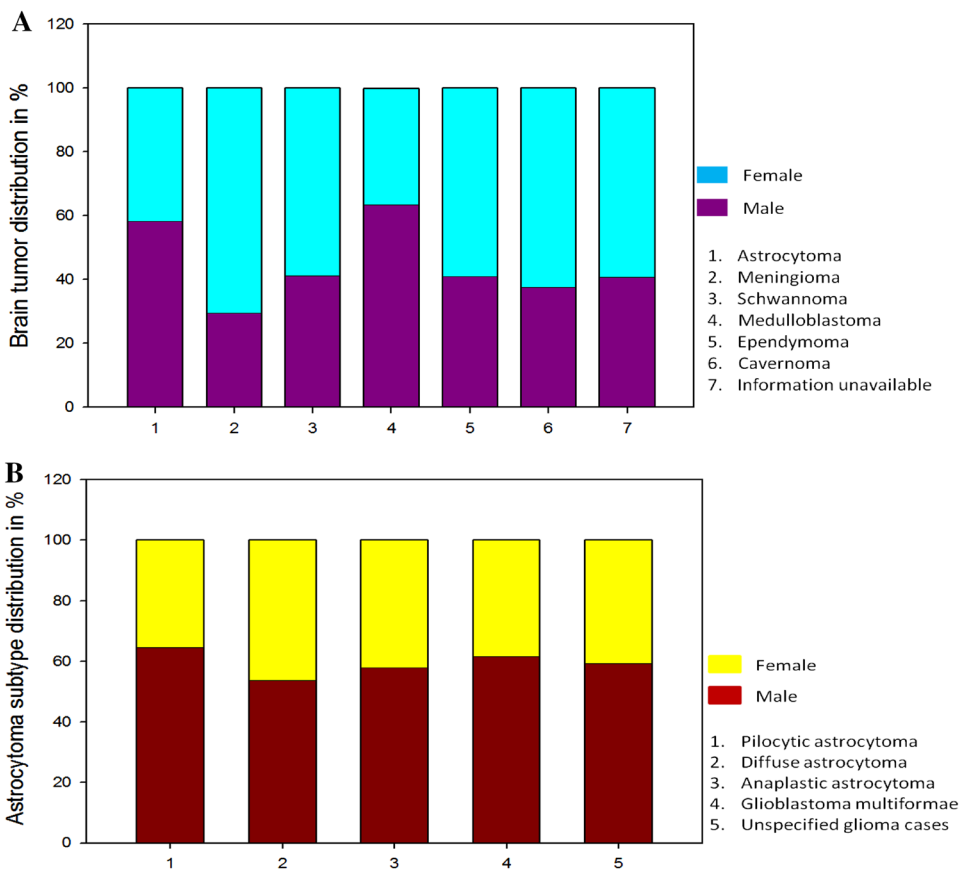

Fig. 3 Gender wise statistical distribution of brain tumors (**a**) and astrocytoma sub types (**b**)

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.

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SIRP Alpha Protein Downregulates in Human Astrocytoma: Presumptive Involvement of Hsa-miR-520d-5p and Hsa-miR-520d-3p

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Abstract Astrocytomas are the most common brain tumors with poor survival in malignant forms. Signal regulatory protein alpha (SIRP alpha) is a transmembrane protein expressed on immune cells and macrophages and is reported to modulate tumor cell phagocytosis. In the present study, we investigated the involvement of miR-520d-5p and miR-520d-3p in regulation of SIRP alpha expression. Here, we report mRNA and protein expression profile of SIRP alpha in 39 surgically resected human astrocytoma tissue samples and 14 control brain tissue samples. Transcript expression pattern was studied by real-time PCR while Western blotting and immunohistochemistry were used to evaluate protein expression. Expression profile of miR-520d-5p and miR-520d-3p was studied by real-time PCR. Computational prediction was employed to analyze the binding of miR-520d-5p and miR-520d-3p for SIRP alpha mRNA. It is evident from preliminary investigation that SIRP alpha transcripts are expressed in control brain tissues, increased in low-grade (grade II) tumor tissues, and decreased with further grade progression ($P < 0.05$). SIRP alpha protein was moderately expressed in control brain tissues but under-expressed in low- and high-grade tissue samples ($P < 0.05$). Immunohistochemistry results further confirmed Western blot outcomes. Computational prediction supplemented with 3′ and 5′UTR targeting analysis and correlation studies reveals that hsa-miR-520d-5p ($P = 0.028$, $R^2 = 0.94$) (95 % CI 0.15 to 0.99) and hsa-miR-520d-3p ($P = 0.027$, $R^2 = 0.94$) (95% CI 0.17 to 0.99) may be the putative microRNAs involved in regulation of SIRP alpha protein expression. Real-time PCR expression profile depicts that mature form of both miRNAs is significantly overexpressed in low-grade (GII) tumor tissue samples compared to control and high-grade (GIII and GIV) tissue samples. MiR-520d-5p and miR-520d-3p were found with expression pattern similar to SIRP alpha transcripts. We show that SIRP alpha protein is under-expressed in low and high grades of astrocytoma patients' tissue samples. Control brain tissues were found to be positive with SIRP alpha protein expression. Real-time PCR expression analysis confirms that miR-520d-5p and miR-520d-3p expression levels were significantly correlated with SIRP alpha transcripts in control, low-grade, and high-grade tissue samples. Computational prediction further evidenced for binding sites of these miRNAs on 3′ and 5′UTR of SIRP alpha transcripts. Taken together, we predict that miR-520d-5p and miR-520d-3p may be having role in the regulation of under-expressed SIRP alpha protein expression.

Keywords Astrocytoma · Glioblastoma · SIRP alpha · miRNA · miR-20d-5p · miR-520d-3p

Introduction

Astrocytomas are the most vivid malignancies of central nervous system. World Health Organization (WHO) has classified astrocytomas in four (pilocytic astrocytoma—GI; diffuse astrocytoma—GII; anaplastic astrocytoma—GII; and glioblastoma multiforme—GIV) grades with reference to its morphological features, angiogenesis, and

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Table 1 Correlation between SIRP alpha protein expression with clinic-pathological parameters ($n = 53$)

Parameters	No. of cases	SIRP alpha protein expression		*P* value
		Positive	Negative	
All cases	53			0.022
Age				
<=45	24	09	15	
>45	29	05	24	
Gender				
Male	26	07	19	<0.001
Female	27	07	20	
WHO Grade				
II	11	00	11	<0.001
III	09	01	08	
IV	19	00	19	
Control	14	13	01	

Expression pattern was categorized into positive and negative on account of Western blotting expression outputs

cellular proliferation status [1]. Present molecular classification schemes have further subclassified glial tumors based on molecular fingerprints, adding value for better therapeutics and to predict prognosis [2–4]. In India, we have reported low median age of patients' diagnosed with glioblastoma multiforme (grade IV astrocytoma) with middle age group being maximum affected [5]. Most commonly followed treatment is surgical resection of tumor followed by temozolomide chemotherapy [6].

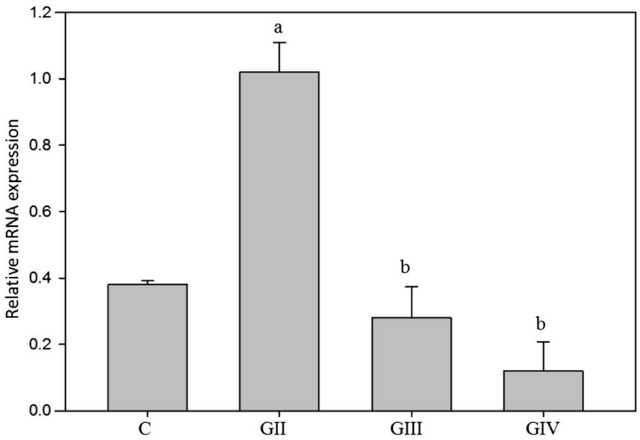


Fig. 1 Real-time PCR expression of SIRP alpha in control, GII, GIII, and GIV tumor tissue samples. Rate of increased transcript expression in GII was statistically significant as compared with control, GIII, and GIV. $P < 0.05$. miR-520d-5p and miR-520d-3p mature form expressions were detected by real-time PCR: mir-520d-3p (Fig. 2) and miR-520d-5p (Fig. 3) expressions were evaluated by real-time PCR. Initially, miR-520d-5p and miR-520d-3p expressions were found to be upregulated from control to GII progression and decreased in higher grade tissue samples. Transcript expression increase of miR-520d-3p and miR-520d-5p in GII was statistically significant as compared with control, GIII, and GIV

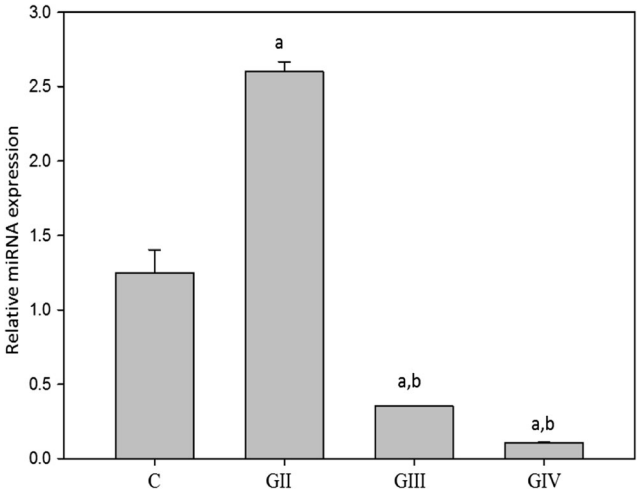


Fig. 2 Real-time PCR expression profile of Hsa-miR-520d-3P in control and three astrocytoma grades. Increase in GII was statistically significant as compared with control, GIII, and GIV ($P < 0.05$). U6 RNA was used as internal control. C = control brain, GII = grade 2, GIII = grade 3, GIV = grade 4

However, despite present clinical advances, median age of patients' diagnosed with glioblastoma is 1–2 years [7]. Present scenario highlights the urgent need to understand the molecular mechanism of glial tumors for therapeutic efficacy.

Signal regulatory protein alpha (SIRP alpha or also known as CD172a) is a transmembrane glycoprotein expressed on myeloid cells and macrophages. Cytoplasmic domain of

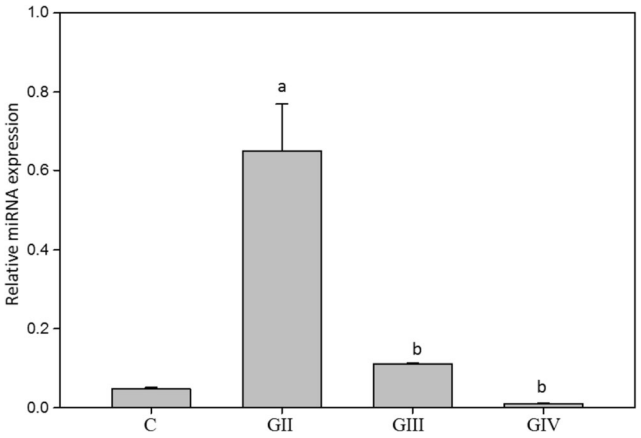


Fig. 3 Real-time PCR expression profile of Hsa-miR-520d-5P in control and three astrocytoma grades. Increase in GII was statistically significant as compared with control, GIII, and GIV ($P < 0.05$). U6 RNA was used as internal control. C = control brain, GII = grade 2, GIII = grade 3, GIV = grade 4. SIRP alpha protein expression in control, low-, and high-grade astrocytoma tissue samples ($n = 45$): SIRP alpha protein expression pattern was evaluated in control ($n = 6$), low-grade (GII, $n = 11$), and high-grade (GIII, $n = 9$, and GIV, $n = 19$) tissue samples. Unlike its mRNA expression pattern, we did not find protein expression in low- and high-grade tissue samples (Figs. 4 and 5). Western blotting results were supplemented by immunohistochemistry analysis (Fig. 6). Control brain sections showed staining while low-grade and high-grade sections were stained negative

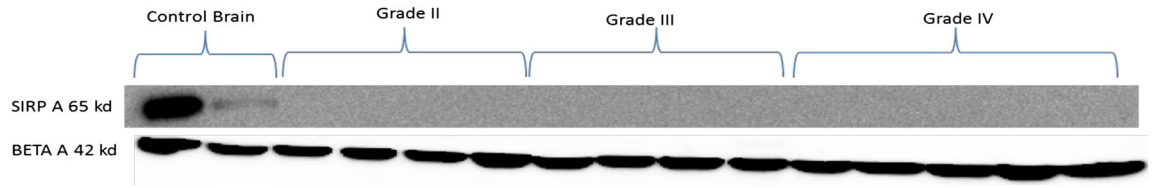


Fig. 4 Western blot for SIRP alpha in control, low- (GII), and high-grade (GIII and GIV) tissue samples ($n = 45$). Beta actin was used as internal control

SIRP alpha contains four tyrosine residues which upon recruitment of SHP1 and SHP2 modulates the signaling pathways, most often in inhibitory fashion [8]. CD47 is reported to serve as ligand for SIRP alpha, and interaction is conserved in human [9], rat [10], and mouse [11]. It plays an important role in phagocytosis, cell-cell interactions, and is viewed as potential target for cancer treatment [12].

Previous studies have shown that SIRP alpha is expressed on RNA levels in astrocytoma cell lines and paraffin-embedded glioblastoma tissue sections [13]; however, these studies did not support findings with relevant control brain tissue samples. Cloning and overexpression studies in U87MG reveal that SIRP alpha modulates signaling pathways in EGFR-dependent ways [14]. Here, we primarily show that SIRP alpha mRNA is overexpressed from control brain to GII progression and further decreased in high-grade (GIII and GIV) tissue samples. However, we could not detect protein expression in low- and high-grade tumor tissue samples. We hypothesized post-transcriptional loop in the form of miRNAs pertinent in regulation of SIRP alpha expression. Computational prediction acquaints putative role of hsa-miR-520d-5p and hsa-miR-520d-3p in regulation of SIRP alpha expression. Subsequently, these miRNAs were found to follow the expression pattern similar to SIRP alpha transcripts. Taken together, our findings reveal that SIRP alpha protein is not expressed in surgically resected tumor tissue samples with presumptive role of hsa-miR-520d-5p and hsa-miR-520d-3p in SIRP alpha post-transcriptional expression.

Materials and Methods

Sample Collection and Processing

Surgically resected astrocytoma tissue samples ($n = 45$) were collected from Krishna Institute of Medical Sciences (KIMS), Secunderabad, India. Tissue samples were clinically and histopathologically diagnosed at pathology department of same hospital. Tissue samples were immediately frozen in liquid nitrogen and stored in −80 °C for further studies. A part of surgically resected tissue was used for RNA isolation, and Western blotting and remaining part were used for immunohistochemistry experiments. Classification of samples was in accordance with WHO [1] classification and shown in Table 1. Epilepsy tissue samples were used as control. Further, two normal brain and 6 epilepsy tissues were collected from the National Institute of Mental Health and Neurosciences (NIMHANS), Bengaluru, India. Informed consent was obtained from the patients. This study was approved by institutional ethics committee. All the participants were completely anonymized.

RNA Isolation and Real-Time PCR

Total RNA was isolated by Trizol reagent (Sigma, cat. No: T9424) according to manufacturer's instructions. Isolated RNA was quantified using nano-drop spectrophotometer (Thermo Scientific, USA). Same pool of RNA was used to evaluate SIRP alpha transcripts and miR-520d-3p and miR-

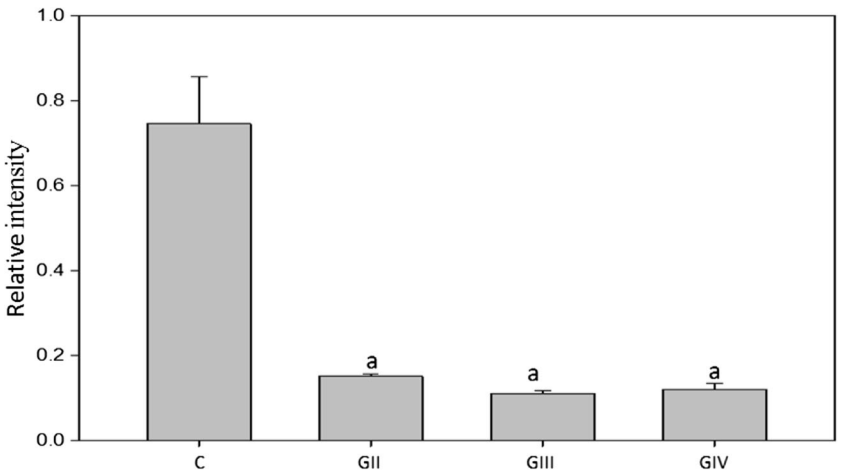


Fig. 5 Densitometric analysis of Western blot for SIRP alpha in control, low- (GII), and high-grade (GIII and GIV) tissue samples ($n = 45$). Decrease in protein expression in low- and high-grade astrocytoma tissue samples was statistically significant compared with control brain tissue samples ($P < 0.05$)

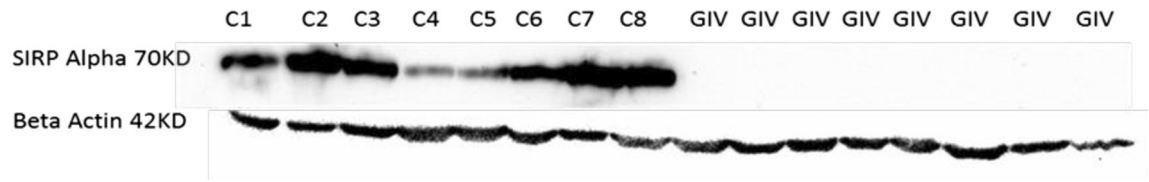


Fig. 6 Expression profile of SIRP alpha in control brain and glioblastoma multiforme tissue lysates. C1, C2 = normal human brain tissue lysate; C3, C4, C5 = temporal lobe epilepsy tissue lysate; C5, C6, C7 = frontal lobe epilepsy tissue lysate; GIV = glioblastoma multiforme tissue lysate

520d-5p. GAPDH was used as internal control for SIRP alpha expression and U6 as internal control for microRNA analysis. Primers sequence used was as follows: SIRP alpha forward primer: 5′-TACAAGGTTGCATGAGCCCGSIRP-3′, alpha reverse primer: 5′-TGGCATACTCCGTGTGGTTGGAPDH-3′, GAPDH forward primer: 5′-AAGGCTGGGGCTCATTTGCAG-3′, reverse primer: 5′-GCAGGAGGCATTGCTGATGATC-3′, U6 forward primer: 5′-GCTTCGGCAGCACATATACTA-3′, U6 reverse primer: 5′-GGAACGCTTCACGAATTTGC-3′, Hsa-miR-520d-5p RT primer: 5′-GTTGGCTCTGGTGCAGGGTCCGAGGTATTCGCACCAGAGCCAACGAAAGG-3′, Hsa-miR-520d-5p mature form forward primer: 5′-GTTGGCTACAAAGGGAAGC-3′, Hsa-miR-520d-3p RT primer: 5′-GTTGGCTCTGGTGCAGGGTCCGAGGTATTCGCACCAGAGCCAACACCCAC-3′, Hsa-miR-520d-5p matured form forward primer: 5′-GTTTGGAAAGTGCTTCTCTTTG-3′, universal reverse primer: 5′-GTGCAGGGTCCGAGGT-3′. cDNA synthesis for SIRP alpha and internal controls was performed using PrimeScript 1st strand cDNA synthesis kit (Takara, cat. No 6110A). Manufacturer's instructions were followed for reaction setup.

For cDNA synthesis of microRNA, equal quantity of RNA was used for accessing SIRP alpha transcript expression. Initially, pulsed RT protocol (65 °C/5 min, 16 °C/30 min [30 °C/30 s] × 60 times, 42 °C/30 s, 50 °C/10 s, 85 °C/5 min) was followed for cDNA synthesis. SYBR green (Takara, cat no: RR041) reagents were used for real-time PCR of SIRP alpha and miRNAs. Analysis was done by $2^{-\Delta\Delta CT}$ method [15]. The efficiency and sensitivity of present protocol have been discussed in previous literature [16–18]

Western Blotting and Imaging

Fifty milligrams of tumor and control brain tissue was homogenized in RIPA buffer supplemented with protease and phosphate inhibitor cocktail (Sigma, cat no.: P8340). Protein concentration was estimated using Bradford reagent (Sigma, cat. No.; B6916). Seventy-five micrograms of estimated protein was mixed with 6× gel loading dye. After boiling for 5 min on water bath, protein was subjected for 10 % SDS polyacrylamide gel electrophoresis followed by Western blotting. Proteins in SDS gel were transferred to nitrocellulose membrane (Millipore) at 30 V for 12 h overnight at 4 °C. SIRP alpha primary antibody (Santa Cruz, Cat no: sc-376884) was diluted to 1:500 in 5 % skim milk solution incubated at 4 °C for 8 h followed by anti-mouse secondary antibody (Sigma, cat no. A9044) for 1 h at room temperature. SIRP alpha protein expression was normalized with beta actin primary antibody (Sigma, cat no. SAB1305567) with 1:5000 dilution. Western blots were imaged using VersaDoc (Bio-Rad, USA) molecular imager.

Immunohistochemistry Analysis

Paraffin-embedded sections of 5-μm thickness were used for immunohistochemistry (IHC) staining. Initially, sections were deparaffinized by heating at 100 °C on slide warmer followed by series of xylene and ethyl alcohol washes. Antigen retrieval was performed with sodium citrate buffer (10 mM sodium citrate, 0.05 % of Tween 20, pH 6.0) in microwave oven for 6, 3, 3, and 3 min. SIRP alpha primary antibody with 1:200 dilution was used. Further steps were followed as per given in user manual of Invitrogen IHC detection kit (cat no: 87-9673).

Computational Prediction of miRNAs Targeting SIRP Alpha Transcript

Micro RNAs (miRNA) putatively targeting to 3′ and/or 5′ UTR of SIRP alpha were screened by use of four tools as

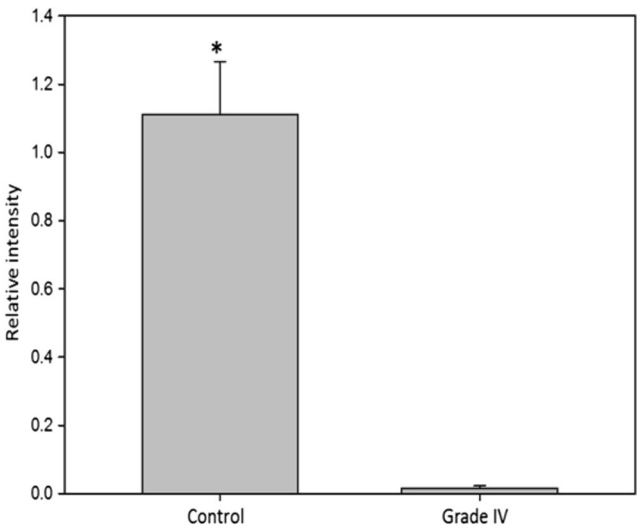


Fig. 7 Densitometric analysis of Western blot image. Overexpression in control brain tissue lysates was statistically significant as compared to glioblastoma multiforme tissue lysates

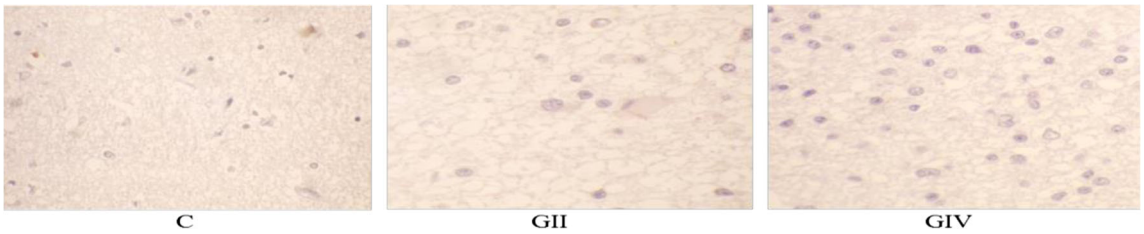


Fig. 8 Immunohistochemistry staining for SIRP alpha in control (*C*), low-grade (*GII*), and high-grade (*GIV*) tissue sections. Control brain sections were found to be positively stained while no significant staining was observed in low- and high-grade sections

target scan (http://www.targetscan.org/) miRanda (http://www.microrna.org/microrna/home.do), miRWalk (http://zmf.umm.uni-heidelberg.de/apps/zmf/mirwalk2/), miRo (http://ferrolab.dmi.unict.it/miro). miRNAs collectively predicted by four of these programs were selected for further analysis. Candidate miRNAs were then screened for SIRP alpha 3′ and 5′UTR binding efficiency by RNA hybrid web server (http://bibiserv.techfak.uni-bielefeld.de/rnahybrid?id=rnahybrid_view_submission).

Statistical Analysis

Statistical analysis was performed using SigmaPlot version 11.0. All results were expressed as mean ± SD. Statistical significance between groups was analyzed by one-way ANOVA. P value <0.05 was considered as statistically significant.

Results

SIRP Alpha mRNA Expression Was Confirmed by Real-Time PCR

SIRP alpha mRNA expression was investigated in 53 tissue specimens. Normal brain and epilepsy tissue samples were used as control. Low-grade tumor tissue (GII) displayed increased expression than control brain and high-grade (GIII and GIV) tissue samples ($P < 0.05$). Expression of SIRP alpha transcripts was found to be decreased with subsequent grade progression (Fig. 1). We have further analyzed expression of miR-520d-5p and miR-520d-3p in astrocytoma tissue samples.

miR-520d-5p and miR-520d-3p mature form expressions were detected by stem-loop real-time PCR.

Mir-520d-3p (Fig. 2) and miR-520d-5p (Fig. 3) expressions were evaluated by stem-loop real-time PCR. Initially, miR-520d-5p and miR-520d-3p expressions were found to be increased from control to GII progression and subsequently decreased in higher grade tissue samples. Elevated mature form expression of miR-520d-3p and miR-520d-5p in GII was statistically significant as compared with control, GIII, and GIV.

SIRP Alpha Protein Expression in Control, Low-, and High-Grade Astrocytoma Tissue Samples (n = 53)

SIRP alpha protein expression pattern was evaluated in control (n = 14), low-grade (GII, n = 11), and high-grade (GIII, n = 9, and GIV, n = 19) tissue samples. Unlike its mRNA expression pattern, we did not find protein expression in low- and high-grade tissue samples (Figs. 4 and 5). Western blotting results were supplemented by immunohistochemistry analysis (Fig. 8). Control brain sections showed staining while low-grade and high-grade sections were stained negative.

Computational Prediction of miRNAs Targeting 3′ and 5′UTR of SIRP Alpha Transcript

miRNAs were screened for putative targeting SIRP alpha transcript. miRNAs collectively predicted by 3 or more programs were considered as strong putative candidates. Further, selected candidate miRNAs were screened for SIRP alpha 3′and 5′ UTR binding efficiency. miR-520d-5p and miR-520d-3p were collectively predicted by 3 programs. 5′ and 3′UTR score analysis strengthens (miR-520d-5p: 3′UTR score: −23.00, 5′ UTR score: −23.00 and miR-520d-3p: 3′UTR score: −29.3, 5′ UTR score: −32.2) putative involvement of these miRNAs in SIRP alpha post-transcriptional regulation (Table 3).

Table 2 Computational prediction of miRNAs targeting SIRP alpha transcripts

Gene	miRNA	Target	minRnada	miRwalk	miRo	Total score
SIRP alpha	hsa-miR-520-5p	1	1	1	1	3
	hsa-miR-520d-3p	1	1	0	1	3

Putative hits were screened by four programs (target scan, miRnada, miRWalk, and miRo). miRNAs predicted by 3 or more programs were considered for further analysis

Table 3 SIRP alpha 3′ and 5′UTR score for miR-520d-5p and miR-520d-3p

miRNA	3′UTR score	5″UTR score
miR520d-5p	−23.0	−23.0
miR520d-3p	−29.0	−32.2

Score value (binding energy) is represented in Kcal/mol

Discussion

In the present investigation, we have attempted to highlight expression profile of SIRP alpha in human astrocytoma and control brain tissue samples. Our studies indicate that SIRP alpha mRNA and protein are expressed in control brain tissues. Increased transcript expression in low grade (GII) was statistically significant compared to control and high-grade tissue samples (Fig. 1). Further Western blotting (Figs. 4 and 6) and densitometric (Figs. 5 and 7) results depict that SIRP alpha protein is expressed in control brain tissue. We did not find protein to follow the mRNA expression outcomes in consequent grade progression. Of 11 GII tissue samples, none was positive for expression. Only one tissue among 9 GIII tissues was found positive and none among 19 GIV tissues was found with detectable levels of expression (Table 1). Immunohistochemistry profile supports Western blotting results. SIRP alpha protein expression was not detectable in low- and high-grade tumor tissue sections while control brain sections were moderately stained (Fig. 8). SIRP alpha is reported to be underregulated in Dalton's lymphoma. Enhanced expression of SIRP alpha and CD47 is reported to revert tumor-associated macrophages to M1 state leading to tumor regression [19]. Cloning and overexpression experiments have shown that SIRP alpha expression leads to SHP2 mediated reduced cell migration and spreading in U87 malignant glioma cells [20].

Further, we have computationally predicted miRNAs involved in regulation of SIRP alpha expression. miR-520d-5p and miR-520d-3p were collectively predicted by 3 or more servers (Table 2). miRNA binds to 3′ and 5′UTR of their targets and mediates post-transcriptional inhibition of target mRNA [21]. So, we have analyzed SIRP alpha 3′ and 5′UTR binding score of screened miRNAs and selected Hsa-miR-520d-5p (5′ UTR score: −23 kcal/mol, 3′UTR score: −23 kcal/mol) and Hsa-miR-520d-3p (5′UTR score: −32.2 kcal/mol, 3′UTR score: −29.3 kcal/mol) as they displayed perceivable binding energy with target mRNA (Tables 2 and 3).

miR-520d-3p and miR-520d-5p mature form expression was validated by real-time PCR. Initially, mature form of both miRNAs increased from control to GII progression and decreased with subsequent high-grade samples (Figs. 2 and 3). Increased expression in GII for miR-520d-5p was statistically significant as compared with control and high-grade tissue samples (Fig. 3). Matured form levels of both miRNAs displayed similar pattern of expression as of SIRP alpha mRNA. Further, we have studied the correlation between expression pattern of SIRP alpha mRNA and predicted mature miRNAs. Hsa-miR-520d-5p was directly correlated with SIRP alpha transcript expression (Fig. 9a, b) with $p = 0.028$, $R = 0.97$. Hsa-miR-520d-3p mature form also directly correlated with SIRP alpha mRNA expression (Fig. 10a, b) with $p = 0.027$, $R = 0.94$. Based on computational targeting reports, mature form expression pattern by real-time PCR, and correlation studies, we presume that miR-520d-5p and miR-520d-3p may be involved in SIRP alpha post-transcriptional regulation.

miR-520d-3p is reported to be downregulated and associated with proliferation, invasion, and migration in gastric

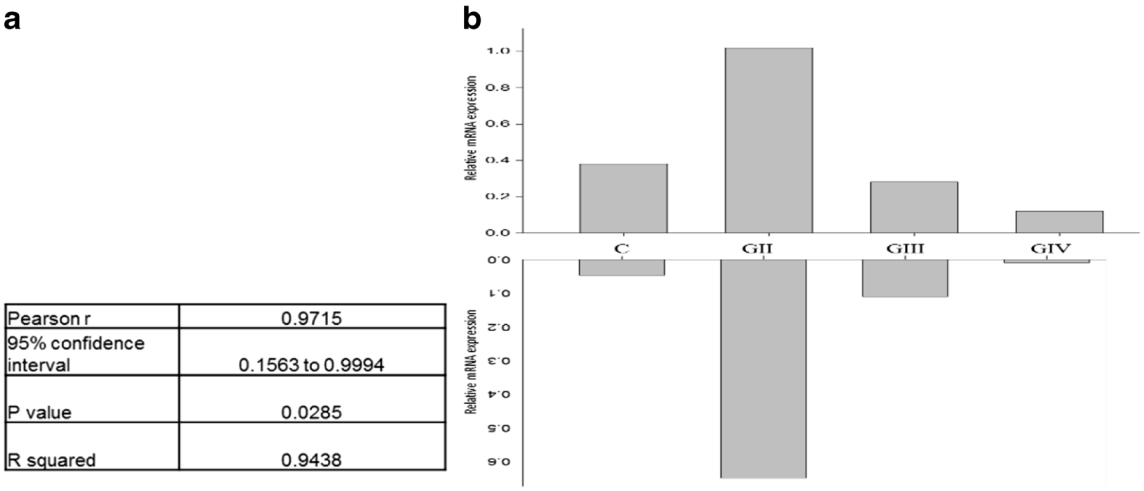
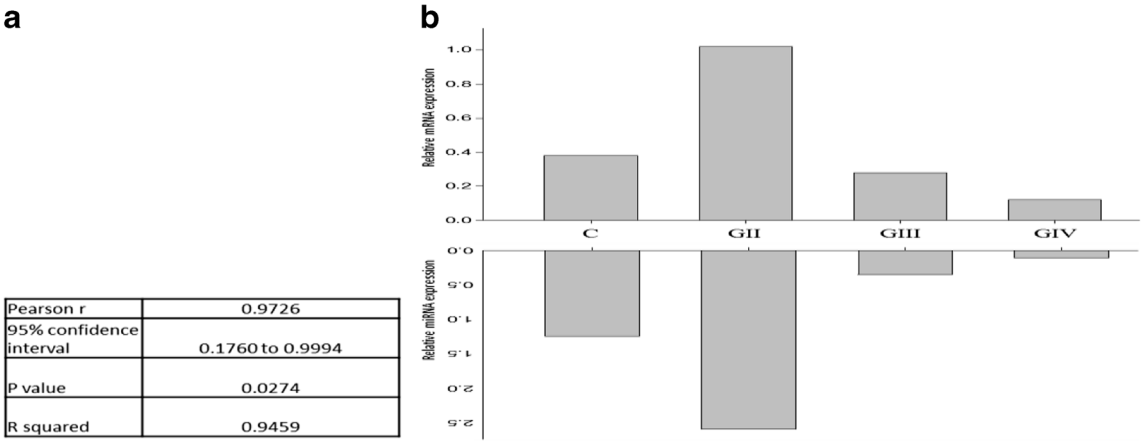


Fig. 9 Correlation analysis of SIRP alpha transcripts and Hsa-miR-520d-5p mature form by real-time PCR in human astrocytoma tissue samples (**a**). **b** Expression patterns were observed with direct correlation ($P = 0.0285$, $R = 0.94$) in cohort of tissue samples



Pearson r	0.9726
95% confidence interval	0.1760 to 0.9994
P value	0.0274
R squared	0.9459

Fig. 10 Correlation analysis of SIRP alpha transcripts and Hsa-miR-520d-3p mature form by real-time PCR in human astrocytoma tissue samples (**a**). **b** Expression patterns were observed with direct correlation ($P = 0.0274$, $R = 0.94$) in cohort of tissue samples

cancer cases [22]. Studies in hepatocellular carcinoma reveal that overexpression of miR-520d-5p is connected with diminished proliferation and invasiveness. miR-520d-5p is reported to mediate its effect through conversion of cancer cells to normal stem cells with maintained p53 expression [23]. Further, miR-520d expression in breast cancer has been associated with diminished proliferation and invasiveness. It is further noted to downregulate metastamiR miR-10b [24]. Recent investigation reveals that miR-10b is upregulated in high-grade astrocytoma tissue samples and correlated with poor prognosis [25]. In astrocytoma, miR-10b is reported to mediate its effect through regulation of proapoptotic genes and cell cycle inhibitors [26] in turn inducing HOXD10 and MMP14 facilitating cell invasion [27]. Additional studies are needed to examine the association of miR-520d-5p and miR-10b in human astrocytoma malignancies. Recently, mice receiving injection of 520d-5p and T98 cells are reported to exist without malignant transformation, thus asserting the possible involvement of miR-520d-5p in glioblastoma progression [28]. Further studies warrant to uncover the underlying mechanisms of tumor suppressive activity of 520d-5p and miR-520d-3p in progression of malignant astrocytoma.

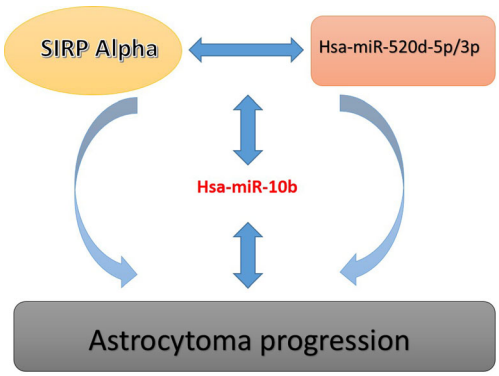


Fig. 11 Role of miR-520d-5p, 520d-3p in SRIP Alpha expression. Possible involvement of this expression module astrocytoma progression in association with miR-10b.

In conclusion, the interesting findings of our investigations are as follows: SIRP alpha transcripts are expressed significantly in GII tissue samples while SIRP alpha protein was not detectable in low- and high-grade astrocytoma tissue samples. miR-520d-5p and miR-520d-5p expression pattern was directly correlated to SIRP alpha mRNA expression, suggesting that the same miRNAs may be involved in SIRP alpha post-transcriptional regulation. As these miRNAs were significantly overexpressed in GII tumor tissue samples as compared to control brain, GIII, and GIV tissue samples, we further propose that miR-520d.-5p and miR-520d-3p could be used as diagnostic markers for low-grade tumors. However, on account of present studies and available literature (Fig. 11), possible mechanisms of SIRP alpha protein in biology of astrocytoma progression need auxiliary investigations.

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Compliance with Ethical Standards

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Conflict of Interest The authors declare that they have no conflicts of interest.

Ethical Standard All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed Consent Informed consent was obtained from all individual participants included in the study.

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Region-Specific Dok2 Overexpression Associates with Poor Prognosis in Human Astrocytoma

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Abstract Astrocytoma is the most frequent malignancies of the brain. Despite present clinical advancements, median survival time in malignant forms remains poor. Downstream of kinase protein 2 (Dok2) is adaptor protein known to modulate the effect of tyrosine kinase. Previously, Dok2 is shown to be marker of poor prognosis in colorectal and gastric cancer, and reduced levels of Dok2 were reported in lung adenocarcinoma and gastric cancer. The aim of the present study was to evaluate prognostic significance of pDok2 expression in surgically resected astrocytoma tissue samples. In the present study, 47 numbers of tissue samples were collected from patients who underwent surgery for astrocytoma. Temporal lobe epilepsy tissues were used as control. Real-time PCR was used to study transcript expression while protein expression was studied by western blotting and immunohistochemistry. The pDok2 expression was categorized as pDok2 positive and pDok2 negative on the basis of intensity of protein expression. This observation was confirmed by two independent pathologists. Control and few GII tissues were used as reference on account for low expression of pDok2 protein. Basic information of patients as anatomic origin of tumor and follow-up details were retrieved from hospital registry. Kaplan-Meier test was used to analyze the association of pDok2 expression and survival outcome in clinical cases. Real-time PCR signifies pDok2 is overexpressed in high-grade (GIII + GIV) tissue samples compared with low-grade (GII) and control brain tissue samples ($p < 0.005$). Western blotting and immunohistochemistry analysis signifies overexpression of pDok2 protein expression in tumor tissue samples as compared with control brain tissues. Clinico-pathological analysis reveals 83% of high-grade astrocytoma (GIII + GIV) and 30% of low-grade (GII) tissue samples which were detected with pDok2 expression. Tumor location was found to be predominant at the frontal and temporal lobes. Survival studies underline prognostic importance of pDok2 protein. Median survival of 20 months was reported with patients with positive pDok2 expression (95% CI 0.083 to 0.49). Taken together, pDok2 protein overexpression is associated with poor prognosis in astrocytoma clinical cases and appears to be an attractive target for therapeutic intervention. Noticeable anatomic origin at the frontal and temporal lobe suggests site-specific role of developmental factors in tumor occurrence.

Keywords Astrocytoma · Dok2 · Glioblastoma · Prognosis · Anatomic origin

Introduction

Astrocytoma constitutes the most common malignancies of central nervous system [1] accounting for 30% of all tumors [2]. In India, we reported patients suffering from astrocytoma have lower median age than western countries [3]. World Health Organization (WHO) classifies astrocytic tumors into four grades (grade I—pilocytic astrocytoma, grade II—diffuse astrocytoma, grade III—anaplastic astrocytoma, and grade IV—glioblastoma multiforme-GBM) on account for morphological features such as cytological atypia, tumor necrosis, and endothelial proliferation which is highly inconsistent in assigning grades and cannot help to determine prognosis in clinical cases [4]. However, recent scheme of classification

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based on the co-expression modules around the most mutated gene such as receptor tyrosine kinases is proposed to accurately assign prognosis in astrocytoma cases [5]. Despite intensive treatment of combined radiotherapy and temozolomide chemotherapy, the median survival age of glioblastoma multiforme patients is around 15 months [6]. This underlines the immediate need to understand more molecular biology of astrocytoma to provide effective therapeutic options.

Downstream of kinase (Dok) is a family of adapter proteins constituting at least 7 members and modulates activity of tyrosine kinases which are the most predominantly mutated family of kinases in cancer [7, 8]. Dok2 is established to associate with Ras-GTPase-activating protein and negatively regulate ERK signaling in B cells [9]. Further, Dok2 is reported to be overexpressed in 66.7% of colorectal cancer clinical samples and may be used as biomarker for prediction of prognosis in patients after surgery [10]. Studies in gastric cancer cases show that Dok2 is overexpressed in 50% of cases and could be of potential use to predict prognosis in patients after curative resection [11]. Studies in lung adenocarcinoma show mutated version of Dok2 promote EGFR-driven tumorigenesis. In EGFR-activated cells, Dok2 was found to be localized at membrane whereas in cells lacking effective EGFR, Dok2 was diffusely distributed in cytoplasm [12].

Little is known about expression profile and prognostic significance of Dok2 in astrocytoma context. Keeping in view the existing findings, here, we have evaluated expression and prognostic significance of Dok2 in surgically resected astrocytoma tissue samples.

Materials and Methods

Human Tissue Samples: Collection, Processing, and Standard Care Followed

In present study, 47 (control brain = 7, grade II = 10, grade III = 11, grade IV = 19) surgically resected astrocytoma tissue samples were collected from Krishna Institute of Medical Sciences (KIMS) Secunderabad. Tissues were immediately snap frozen with liquid nitrogen and stored in −80 °C for further molecular analysis. The grade of astrocytoma samples was confirmed by criterion as enhanced cellularity, mitotic index, and nuclear proliferation specified by World Health Organization (Table 1). Initially, location of tumor was confirmed by magnetic resonance imaging (MRI) and was broadly categorized in the frontal, parietal, temporal, and occipital lobes in the brain. In case of tumors with overlap in two lobes, area with maximum tumor appearance was selected. For example, tumor with parieto-occipital appearance with maximum location in parietal lobe was regarded as parietal origin. Age/sex of subjects and follow-up details were maintained at hospital registry. Temporal lobe epilepsy tissues were used as control. A part of tissue samples were used to isolate RNA for western blotting while another part was used for paraffin embedding which further used for immunohistochemistry studies. Tumor tissue was removed by near total and subtotal resection. Standard treatment includes radiotherapy and concomitant chemotherapy usually with temozolomide. Postoperative scan was monitored for every 3 and 6 months initially and then yearly to trace tumor recurrence. The studies are approved by the KIMS Foundation and Research Centre (KFRC) and KIMS Ethical Committee and were supported by informed consent form from the patients/close relatives. Institutional ethical guidelines were followed. All the subjects were completely anonymized.

Table 1. Association of pDok2 protein expression with clinicopathological parameters evaluated in patients

Parameters	No. of cases	pDok2 protein expression		*p* value
		Positive	Negative	
All cases	47			0.083
Age				
≤45	18	11	7	
>45	29	19	10	
Gender				0.257
Male	30	19	11	
Female	17	11	6	
WHO grade				<0.001
II	10	3	7	
III	11	9	2	
IV	19	16	3	
Control	7	2	5	

RNA Isolation and Real-Time PCR

RNA was isolated from frozen tissuee samples by TRIzol method. RNA was quantified by NanoDrop spectrophotometer (Thermo Scientific). Five micrograms of RNA was used for C-DNA synthesis by PrimeScript 1st-strand C-DNA synthesis kit (TaKaRa, cat. No 6110A) following the manufacturer's instructions. SYBR green (TaKaRa, cat.no. RR82LR) was used for real-time PCR (Applied Biosystems, USA) analysis with equal concentration of C-DNA for each experiment. Primers were designed manually, and GAPDH gene was used as internal control (DOk2 forward primer: TTTGGGCG GGACAAGGTAAC, reverse primer: TCCAGGGC CAAGAAGATCTC, Tm 60.5 °C; GAPDH forward primer: AAGGCTGGGGCTCATTTGCAG, reverse primer: GCAGGAGGCATTGCTGATGATC, Tm 60 °C). Samples were run in duplicates and for 35 cycles. Gene expression pattern was analyzed relative to internal control by $2^{-\Delta\Delta CT}$ method [13].

Western Blotting

Approximately 100 mg of tumor tissue was homogenized in RIPA buffer. Protease inhibitor cocktail (Sigma, cat no.: P8340) was added as per manual instructions. Protein concentration was estimated using Bradford method, and 75 μg of protein was added with SDS sample buffer (0.125 M Tris pH 6.8, 4% SDS, 20% glycerol, 0.2 M DTT, 0.02% bromophenol blue). Protein extracts were then kept in boiling water bath for 5 min and used for loading in 10% SDS-PAGE. This was followed by transfer of protein from SDS gel to nitrocellulose membrane (Millipore) overnight in Towbin (0.048 M Tris, 0.039 M glycine, 20% methanol, 0.00375% SDS) buffer at 25 V. Five percent nonfat skimmed milk powder in TBST was used as blocking solution. Then, the blots were incubated with primary pDOk2 (Sc-13952, diluted to 1:500 in % skimmed milk solution) antibody. Blots were probed with alkaline phosphatase conjugated antirabbit secondary antibody (Sigma, cat. No. A9919, Diluted to 1: 50,000 in TBST) for 1 h at room temperature. Blots were developed by BCIP/NBT solution (Sigma, cat. No. B6404) in ALP buffer (100 mM Tris, 100 mM NaCl, 50 mM $MgCl_2$) at room temperature.

Immunohistochemistry

Immunohistochemical staining was performed using pDok2 primary antibody (1:200 dilution, Santa Cruz Biotechnology, USA). Paraffin-embedded sections (5 μm) were prepared from surgically resected tissue using Leica microtome. Sections were dewaxed by heating on slide warmer (Yorco slide warmer) at 100 °C for 10 min. This step followed washes by xylene and series of alcohol for rehydration. Antigen retrieval was performed with citrate buffer (10 mM sodium citrate, 0.05% of Twin20, pH 6.0) in microwave oven for 15 min. After cooling slides at room temperature, were incubated with primary pDok2 antibody. Further steps were followed as given in the user manual of Invitrogen IHC detection kit (cat no: 87-9673).

Statistical Analysis

Statistical analysis was performed using SigmaPlot version 11.0. All results were represented as mean ± SD obtained from individual patients of each grade and were compared with control. The significant differences of the data were determined using one-way ANOVA and log-rank test. Values of $P < 0.05$ were considered as statistically significant.

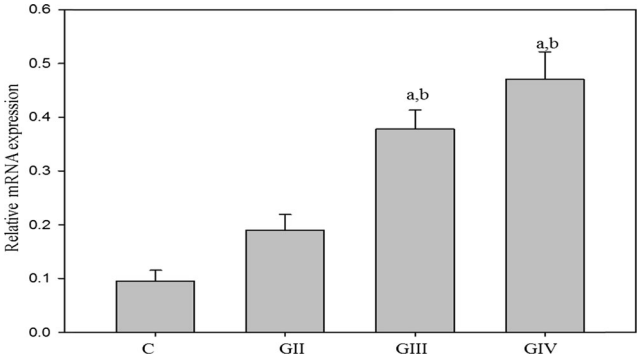

Fig. 1. Real-time PCR for Dok2 transcripts in control, grade II, grade III, and grade IV tissue samples. *a* increased transcript expression in GIII and GIV tumor tissue was statistically significant as compared with control (*a*) brain tissues ($p < 0.05$), *b* increased transcript expression in GIII and GIV tumor tissue was statistically significant as compared with GII (*b*) tissues ($p < 0.05$). *C* control brain, *GII* grade II, *GIII* grade III, *GIV* grade IV

Results

Overexpression of Dok2 Transcripts in Human Astrocytoma

Dok2 transcripts were found to be increased significantly in high-grade astrocytoma tissue samples as compared with control brain tissues. GAPDH was used as internal control. The analysis was performed in total 47 tissue samples (07 control Brain, 10 GII, 11 GIII, 19 GIV tissue samples). Increased levels of transcripts in high-grade astrocytoma (GIII and GIV) tissue samples were significant as compared with low-grade (GII) and control brain tissue samples (Fig. 1).

pDok2 Protein Expression Significantly Altered in Human Astrocytoma Tissue Samples

pDOk2 protein levels were significantly expressed ($p < 0.005$) in high-grade astrocytoma (GIII, $n = 11$, and GIV, $n = 19$) tissue samples compared with low-grade (GII, $n = 10$) and control ($n = 07$) brain tissue samples. Of 7 control brain tissue samples, only 2 were found to express pDOk2 protein moderately (Fig. 2). Densitometric analysis (Fig. 3) reveals positive expression of protein in grade IV was statistically significant as compared with control brain ($p < 0.001$) and grade II ($p = 0.011$) tissue samples. Similarly, positive expression in GII and GIII was statistically significant as compared with

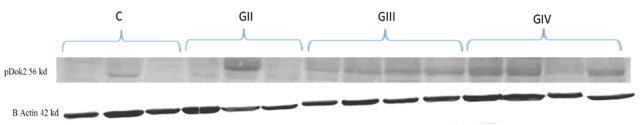

Fig. 2. Western blot for pDok2 protein expression in control, low-grade (GII), and high-grade (GIII and GIV) tissue lysates. Expression in high-grade tissues was found to be significantly higher as compared to low-grade and control brain tissues. Beta actin was used as internal control. *C* control brain, *GII* grade II, *GIII* grade III, *GIV* grade IV

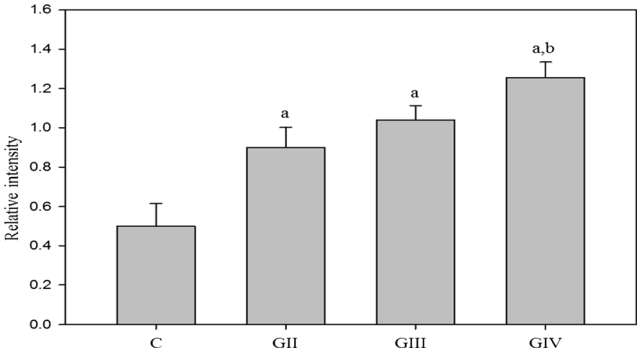

Fig. 3. Densitometric analysis for pDok2 protein expression in control, grade II, grade III, and grade IV tissue samples. Beta actin was used as internal control. *a* increased pDok2 protein expression in GII, GIII, and GIV tumor tissues were statistically significant as compared with control (*a*) brain tissue ($p < 0.05$), *b* increased expression in GIV tissue was statistically significant compared with control and GII (*b*) tumor tissue ($p < 0.05$). *C* control brain, *GII* grade II, *GIII* grade III, *GIV* grade IV

control ($p = 0.005$ and $p < 0.001$, respectively). Further, western blotting outcomes were supported by immunohistochemistry experiments. High-grade astrocytoma (GIV) sections were found to be positively stained with pDOk2 as compared with low-grade (GII) and control tissue sections (Fig. 4).

pDok2 expression was significantly correlated with progression of tumor grade ($p < 0.001$). Age ($p = 0.083$) and gender ($p = 0.257$) of participants evaluated were not significantly associated with degree of pDok2 expression. Among the tissue samples used (control = 7, GII = 10, GIII=11, GIV = 19), 16/19 GIV, 9/11 GIII, and 3/10 GII samples were found to express pDok2 protein. Of 7 control brain samples, only 2 were detected with moderate pDOk2 expression. Overall, 25/30 high-grade (GII + GIV) samples were reported to be positive with pDok2 protein (Table 1).

Anatomic Origin of Tumor and Follow-Up Details

Initially, tumor in the brain was located by MRI imaging. In the present study, 17 females and 30 male subjects (gender ratio 1.76) were included (Table 2). Tumor was found to be located for 13 cases in the frontal lobe, 05 cases in the parietal lobe, 17 cases in the temporal lobe, and 05 cases in the occipital lobe (Table 3). Follow-up of patients was monitored for initial interval of 3 and 6 months and then per year.

pDok2 Expression and Patients' Prognosis

To evaluate the association of pDok2 protein expression with patients' prognosis, expression pattern was categorized into two groups with the help of pathologists: pDok2 positive and pDok2 negative. This categorization was based on account for degree of pDOk2 protein expression and confirmed by two independent observations. Control and few GII tissues were used as reference on account for observed low expression of pDok2 protein. Log-rank test was used to evaluate the effect of pDok2 expression on prognosis of astrocytoma patients. Of 47 cases, follow-up details were not available for 5 patients. As shown in Fig.5, total 42 cases were studied. Positive expression of pDok2 was displayed in 26 cases while negative expression in 16 cases. Significant difference was observed among pDok2-positive and pDok2-negative group (Fig. 5b) with $p = 0.0005$, chi-square = 12.8, and 95% CI 0.083 to 0.49. Patients with positive pDok2 expression were found to have median survival age of 20 months.

Discussion

In present investigations, we demonstrate for the first time the expression profile and prognostic importance of pDok2 expression in human astrocytoma tissue samples. Real-time PCR (Fig.1) signifies pDok2 transcripts are significantly expressed in high-grade (GIII + GIV) tissue samples as compared with low-grade (GII) and control brain tissues ($p < 0.005$). Western blotting (Figs. 2 and 3) and immunohistochemistry (Fig.4) analysis signifies overexpression of pDok2 protein in surgically resected tissues as compared with control brain tissues. High-grade (GIII + GIV) tissue samples were found considerably express pDok2 protein as compared with control and low-grade (GII) tissue samples ($p < 0.001$). Clinico-pathological analysis reveals degree of pDok2 expression was meaningfully correlated with grade progression ($p < 0.001$) and not with age ($p = 0.083$) or sex ($p = 0.257$) of patients (Table 1). We have

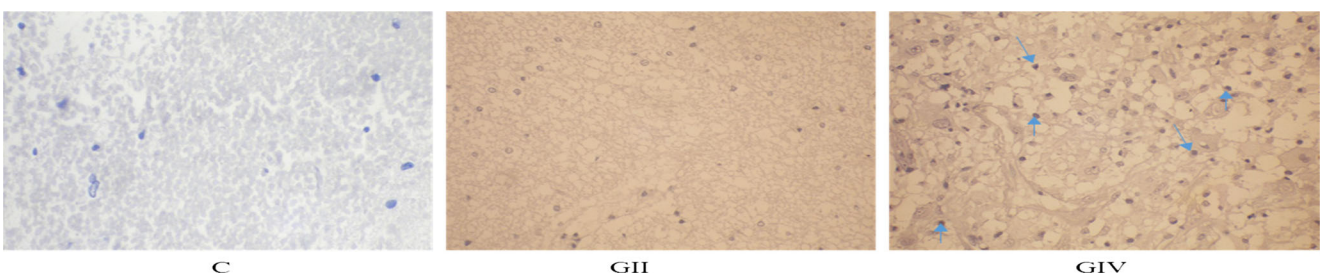

Fig. 4. Immunohistochemistry analysis for pDok2 protein in control, low-grade (GII), and high-grade (GIII) astrocytoma tissue samples. Grade IV section was found with increased cellularity and cells with overexpressing pDok2 as compared with control brain. Cell with positive staining are marked by *arrow*. *C* control brain, *GII* grade two, *GIV* grade IV

Table 2. Anatomic origin of tumor and follow-up details

Patient sr. no.	Age/sex	Tumor type	Location of tumor	Follow-up time (in months)
1	51/M	Grade III	Frontal	25
2	48/M	Control	Temporal	31
3	38/M	GBM	Temporal	35
4	55/F	Grade II	Parietal	20
5	46/M	GBM	Frontal	32
6	44/M	Control	Temporal	38
7	29/F	Grade III	Temporal	23
8	58/M	Grade II	Temporal	24
9	42/M	GBM	Frontal	31
10	49/M	Grade III	Parietal	29
11	52/M	GBM	Temporal	15
12	34/F	GBM	Frontal	40
13	37/M	Grade II	Temporal	41
14	49/F	GBM	Frontal	41
15	50/M	Grade II	Temporal	N.A.
16	56/F	GBM	Temporal	12
17	25/M	GBM	Temporal	14
18	61/M	Grade III	Frontal	20
19	41/M	Grade III	Temporal	19
20	62/F	GBM	Frontal	29
21	44/M	Grade III	Parietal	N.A.
22	53/F	GBM	Frontal	24
23	56/M	Control	Temporal	29
24	35/F	GBM	Temporal	15
25	57/M	Control	Temporal	26
26	31/F	Grade III	Frontal	24
27	51/M	GBM	Temporal	13
28	28/F	Grade II	Occipital	16
29	30/M	Grade II	Temporal	24
30	55/F	GBM	Frontal	29
31	46/M	Control	Temporal	N.A.
32	15/F	Grade II	Temporal	9
33	47/M	GBM	Temporal	7
34	52/M	GBM	Temporal	9
35	41/F	Grade II	Parietal	24
36	55/M	Grade III	Frontal	10
37	58/M	Grade II	Parietal	N.A.
38	15/M	GBM	Occipital	11
39	59/F	Grade III	Occipital	15
40	60/M	GBM	Occipital	12
41	61/F	Grade II	Occipital	17
42	65/M	GBM	Frontal	N.A.
43	46/M	Grade III	Frontal	28
44	35/F	Grade III	Temporal	24
45	52/M	Control	Temporal	38
46	39/F	GBM	Temporal	N.A.
47	48/M	Control	Temporal	35

N.A. not available

Table 3. Distribution of tumor locations on the basis of anatomic origin in the brain

Lobe Grade	Frontal	Parietal	Temporal	Occipital
Grade II	01	03	04	02
Grade III	04	02	04	01
Grade IV	08	00	09	02
Total	13	05	17	05

further seen the prognostic significance of pDok2 expression. Patients with pDok2 expression were found with significantly lower survival than with under-expressed pDok2 ($p = 0.0005$) (Fig. 5a, b, Table 2).

Inconsistence with our results, previous studies show that pDok2 is upregulated in moderately differentiated colorectal adenocarcinoma and can serve as biomarker for poor prognosis in patients after curative restriction [14]. Studies in gastric cancer reports Dok2 to be upregulated in 50% gastric cancer samples and could be used as marker for prediction of prognosis in clinical studies [15]. Overexpressed Dok2 have been shown to promote angiopoietin-mediated cell migration by influencing actin cytoskeleton rearrangement [16]. Angiopoietin1/angiopoietin2 balance was shown to have prognostic value in astrocytoma patients [17]. However, the association of Dok2 and angiopoietin signaling is elusive in astrocytoma progression. Dok family of protein appears to be functionally conserved among the lineage. Dock, a homolog of Dok family of proteins, has been shown to play a role in axonal guidance in drosophila [18].

Mice with knocked-out Dok1 (homolog of Dok2) and Dok2 developed myeloproliferative disorder resembling myeloid leukemia in human [19]. Studies in lung cancer [20] have reported tumor suppressive function of Dok2. Loss of Dok2 in ovarian cancer [21] has been shown to induce chemotherapy resistance in response to treatment by decreasing apoptosis levels. Mutational analysis of Dok2 in leukemia [22] reveals no detectable somatic mutations. This suggests another mechanism as deletion of locus may be prevalent. Further studies are needed to ascertain the multiple components of Dok2 and its effector mechanisms.

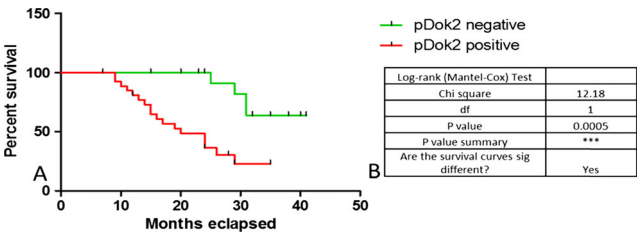

Fig. 5. Kaplan-Meier survival curve (**a**) with log-rank test (**b**) for patients with positive (*red line*, $n = 26$) and negative (*green line*, $n = 16$) pDok2 expression. Median survival of 20 months was reported with patients with high pDok2 expression (95% CI 0.083 to 0.49)

Further, we have seen anatomic origin of tumor locations in the brain. In total, 13 tumors were located at the frontal lobe, 05 in the parietal lobe, 17 in the temporal lobe, and 05 in the occipital lobe (Table 3). We found predominantly the frontal and temporal lobe as anatomic site for glioblastoma multiforme. Of total 19 glioblastoma multiforme cases we studied, in 08 cases, tumor was located at the frontal lobe, in 09 cases at the temporal lobe, and in 02 cases at the occipital lobe. Overall, we observed nonuniform distribution of tumor. It highlights possible role of transcription or developmental factors involved at particular locations present findings are in agreement with previous studies regarding dominated occurrence at the frontal and temporal lobes [23].

In conclusion, present study shows that pDok2 was found to express significantly in 84% of GIV astrocytoma tissue samples. In total, 83% of high-grade astrocytoma (GIII + GIV) and 30% of low-grade (GII) tissue samples were detected with pDok2 expression. Clinico-pathological and survival studies underline the use of pDok2 protein as marker for prediction of prognosis in astrocytoma patients.

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Compliance with Ethical Standards The studies are approved by the KIMS Foundation and Research Centre (KFRC) and KIMS Ethical Committee and were supported by informed consent form from the patients/close relatives.

Conflict of Interest Authors declare that there are no conflicts of interest.

Ethical Standard All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

Informed Consent Informed consent was obtained from all individual participants included in the study.

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Retrospective clinical analysis and pathological significance of biomarkers for astrocytoma progression

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PRIMARY SOURCES

1	Ravindra Pramod Deshpande, Y.B.V.K Chandra Sekhar, Manas Panigrahi, Phanithi Prakash Babu. "SIRP Alpha Protein Downregulates in Human Astrocytoma: Presumptive Involvement of Hsa-miR-520d-5p and Hsa-miR-520d-3p", Molecular Neurobiology, 2016 Publication	%14
2	Ravindra Pramod Deshpande, Y. B. V. K. Chandra Sekhar, Manas Panigrahi, Phanithi Prakash Babu. "Region-Specific Dok2 Overexpression Associates with Poor Prognosis in Human Astrocytoma", Molecular Neurobiology, 2016 Publication	%13
3	Deshpande, Ravindra Pramod, Deepak Babu, Manas Panigrahi, Y. B. V. K. Chandra Sekhar, and Phanithi Prakash Babu. "Brain tumors incidences and a retrospective clinical analysis from a tertiary hospital in India", Journal of Neuro-Oncology, 2016.	%7