Chapter 2
Molecular Pathology of Glioblastoma- An Update

Vani Santosh, Palavalasa Sravya, and Arimappamagan Arivazhagan

Abstract Glioblastoma, the most common primary brain malignancy, has piqued the interest of researchers for decades. As a result, it is one of the most studied brain malignancies. Advancement in technology in recent years has had a tremendous impact on the understanding of this dreaded disease. Deepening insight into its molecular pathology has brought about a paradigm shift in the knowledge of this disease. The WHO has made significant changes in the classification of glioblastoma with emphasis on the molecular changes, thus advocating a histomolecular diagnosis, as opposed to the purely histological diagnosis which was the gold standard until recently. The molecular diagnostics aid the decision making in the management of the disease. This chapter discusses the histomorphology of glioblastoma, new WHO classification of glioblastoma, recent molecular contributions of various research groups leading to changing concepts and also the less explored avenues like intra-tumor heterogeneity and tumor recurrence in glioblastoma.

Keywords Glioblastoma • WHO 2016 • Molecular pathology • IDH1 • MGMT • Recurrence

2.1 Introduction to Adult Diffuse Gliomas and Glioblastoma with Emphasis on Changing Concepts

Gliomas are the most common primary brain tumors in adults and are the focus of research in neuro-oncology. Until recently, they have been grouped together according to what was believed to be their cell of origin. Hence, they were classified as astrocytic, oligodendroglial, oligoastrocytic and ependymal tumors. Initially, the cell
of origin was predicted solely based on the morphological similarities of the neo-
plastic cells to the different glial cells. However, with advancing technology, the
picture of molecular landscape of gliomas has become sharper and the family trees
have been re-drawn. It is now understood that the various diffusely infiltrating gli-
omas are more nosologically similar to each other than to the circumscribed gliomas
sharing similar cellular morphology.

Thus, according to the latest WHO 2016 classification, adult diffuse gliomas,
whether astrocytic or not, now fall under one category and comprise the WHO grade
II and grade III astrocytoma, grade II and grade III oligodendroglioma, grade II and
grade III oligoastrocytoma-‘Not Otherwise Specified(NOS)’ and grade IV glioblas-
toma . Previously, pediatric gliomas were grouped along with their adult counter-
parts, despite the known differences in their biological behavior. Increasing insights
into the molecular aberrations in pediatric gliomas have enabled sharper demarca-
tion in the subtypes. One such group defined in the new classification is the diffuse
midline glioma, H3 K27 M mutant. Overall, the most notable change in the way
gliomas are now viewed at is the integration of molecular markers in defining the
entities.

Diffuse gliomas are potentially malignant or overtly malignant tumors, glioblas-
toma being the most aggressive of all. Its inexorable progression and the inevitabil-
ity of death it brings about within 14–16 months of diagnosis despite the best
available treatment makes it the nightmare of patients, clinicians and researchers
alike. Glioblastoma has plagued the minds of researchers ever since the entity came
to be known. Diagnosis of this tumor is not so much of a challenge, but predicting
its clinical behavior is. This tumor is so varied in its composition that heterogeneity,
both inter and intra tumor, is one of the characteristic features of glioblastoma. Prior
to the advent of technologies to decipher the molecular makeup of glioblastoma,
histopathology was the only modality available to characterize the tumor and its
variants.

In glioblastoma, the heterogeneity is such that if each pattern were to be consid-
ered a variant, there would be many variants which are of doubtful prognostic sig-
nificance and which would not, with the present knowledge, aid the prediction of
clinical and biological behavior. Hence, the WHO has identified only those patterns
as variants which would throw light on possible clinical course. WHO 2007 classi-
fication had identified Glioblastoma as one codified tumor entity with two variants-
Gliosarcoma and Giant cell glioblastoma.

With the advent of molecular profiling which picked up speed in the last decade,
the understanding of the tumor has moved up by several notches and the latest WHO
2016 classification now identifies two entities of glioblastoma based on mutational
status of IDH (Isocitrate dehydrogenase) gene-IDH wild type glioblastoma and IDH
mutant glioblastoma. A new variant of IDH wild type glioblastoma also has been
penned down, which is epithelioid glioblastoma. Glioblastoma with primitive neu-
ronal component has been described as a pattern.

It is evident that there was an enormous inflow of molecular data which com-
pelled the scientific community to reconsider the approach to gliomas. Discoveries
of note are 1p and 19q codeletion (oligodendroglioma specific), IDH point muta-
tions, ATRX mutations, TP 53 mutations, TERT mutations, EGFR amplifications, PTEN mutations and others. This flood of molecular findings has brought about a revolution in the diagnosis and prediction of clinical behavior of gliomas, including glioblastoma. The ISN Haarlem guidelines have played a central role in bringing about this change.

2.2 ISN Haarlem Guidelines and Evolution of WHO 2016

The inadequacy of the WHO 2007 guidelines in prognosticating the patient’s response to treatment and the inter-observer variability which was prevalent when WHO 2007 classification failed to guide the pathologists in arriving at an unequivocal diagnosis, instigated the International Society for Neuropathology to convene at Haarlem, Netherlands in order to discuss the need for incorporation of pertinent molecular discoveries into the existing diagnostic criteria and classification. This crucial meeting, called the “WHO’S NEXT” has set the stage for the new WHO classification. However, the WHO authorized an ‘update’ of the WHO 2007 4th edition but not the release of a 5th edition. This update features predominantly a combination of morphology and genetics resulting in a major restructuring in the classification of several brain tumour entities, the gliomas in particular.

The ISN Haarlem guidelines suggested that a “layered diagnosis with histological classification, WHO grade and molecular information listed below to derive an integrated diagnosis” be made routinely (Louis et al. 2014) (Table 2.1).

With this, the picture became clearer and several unresolved issues have successfully attained a greater resolution.

2.3 WHO 2007 Classification Versus 2016 Classification w.r.t Glioblastoma (Table 2.2)

Following the suggestions made by ISN Haarlem consensus and taking into account the advancement in understanding of molecular pathogenesis, the WHO 2016 has made significant changes in the classification of gliomas. One major change is the regrouping of the gliomas as ‘diffuse astrocytic and oligodendroglial tumors’,
other astrocytic tumors’, ‘ependymal tumors’ and ‘other gliomas’ as opposed to the WHO 2007 that includes; ‘astrocytic tumors’, ‘oligodendroglial tumors’, ‘oligooastrocytic tumors’, ‘ependymal tumors’ and ‘other neuroepithelial tumors’. Another significant change in WHO 2016 is the inclusion of entity defining molecular information in the classification. Now, various entities are defined by their molecular signatures.

Both these major changes apply to Glioblastoma, too. It is now grouped with diffuse gliomas which are now believed to arise from a common bipotential precursor cell/ a neural stem cell which undergoes sequential mutations that directs its evolution to the different types of diffuse glioma. A new entity namely “Glioblastoma-IDH mutant” is recognized. This change is guided by accumulating evidence that IDH mutant glioblastoma confers a significantly better prognosis than IDH wild type glioblastoma.

2.4 Glioblastoma: Gross Pathology, Histomorphology and the New Definitions

Even though histomolecular approach has now taken over pure histological approach for brain tumor diagnosis, it must, however, be re-iterated that histology is the key entry point, which is especially true for glioblastoma. The holistic understanding of glioblastoma is only complete when one is well versed with its histomorphology. Few human malignancies display the heterogeneity which glioblastoma exhibits. The cellular composition is varied and as a result, there are numerous histological patterns of glioblastoma which have been described in great detail.

Glioblastomas are disproportionately large for the duration of symptoms with which the patient presents. The tumor, in no time, completely infiltrates an entire lobe. In fact, one of the earliest descriptions (1928) of treatment for glioblastoma was that of hemispherectomy by neurosurgeon Walter Dandy, despite which the patients succumbed due to contralateral hemisphere involvement (Bahuleyan et al. 2013). This vignette clearly shows how aggressively the tumor grows.

### Table 2.2 Differences between WHO 2007 (Louis et al. 2007) and WHO 2016 (Louis et al. 2016) classification w.r.t glioblastoma

<table>
<thead>
<tr>
<th>WHO 2007</th>
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<tr>
<td><strong>Astrocytic tumors:</strong></td>
<td><strong>Diffuse gliomas:</strong></td>
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<tr>
<td>Glioblastoma ICD code 9440/3</td>
<td>Glioblastoma IDH wild type ICD code 9440/3</td>
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<tr>
<td>Giant cell glioblastoma ICD code 9441/3</td>
<td>Giant cell glioblastoma ICD code 9441/3</td>
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<td>Gliosarcoma ICD code 9442/3</td>
<td>Gliosarcoma ICD code 9442/3</td>
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<td></td>
<td>Epitheloid glioblastoma ICD code 9440/3</td>
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<td>Glioblastoma NOS ICD code 9440/3</td>
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^NOS Not otherwise specified
While a vast majority of glioblastomas show such aggressive biological and clinical behaviour, a small group of glioblastomas present with long duration of symptoms and usually occur in younger adult patients. These tumors are believed to evolve from a lower grade astrocytoma to glioblastoma over a long duration. These are referred to as “clinico-pathologically defined secondary glioblastomas” whereas the aggressive glioblastomas, without evidence of a less malignant precursor lesion, are called “clinico-pathologically defined primary glioblastomas or de novo glioblastomas”. The secondary glioblastoma is found to have a significantly longer survival than the primary glioblastoma. The discovery of IDH mutations provided the reason for this dramatic difference. It was later understood that the clinico-pathologically defined secondary glioblastoma corresponds very well to the IDH mutant glioblastoma.

The lesions, though usually unilateral, may present as supratentorial bilateral tumor mass due to extension along the myelinated structures, especially the corpus callosum and the commissures (Fig. 2.1a, b). Multifocal glioblastoma as visualized on radiological imaging is not unusual. Whether the seemingly independent multi-

**Fig. 2.1** Gross morphology of glioblastoma. (a) Depicts tumor in the right frontal region showing diffuse infiltration of the white matter, overlying grey matter, including cingulate gyrus and spreading across the corpus callosum (black arrows). Another small tumor nodule is noted beyond the discernible margin of the main tumor mass indicating spread (white arrow). Tumor shows a variegated appearance with discoloration indicating necrosis and hemorrhage. (b) Shows tumor in the right frontal region diffusely infiltrating the parenchyma resulting in compression of right lateral ventricle and shift of midline structures with compression of contralateral lateral ventricle (black arrows). (c) Depicts a coronal slice of a resected glioblastoma tumor showing tumor located superficially in the white matter (black arrow) with areas of hemorrhage and with spread into the overlying grey matter (white arrow)
ple lesions are truly multifocal is to be determined. Truly multifocal gliomas are usually seen in inherited neoplastic syndromes. But outside of this setting, true multifocal gliomas are relatively rare, with studies reporting about 2.4% glioblastomas to be truly multifocal with different foci showing different clonality (Batzdorf and Malamud 1963).

Most glioblastomas arise from the white matter of cerebral hemispheres but sometimes, they may be largely superficial (Fig. 2.1c). They are diffusely infiltrating, peripherally greyish with central areas of yellowish necrosis. There may also be extensive hemorrhages and macroscopic cysts containing liquefied necrotic tissue.

Microscopically, glioblastoma appears as a highly cellular tumor, composed of a wide variety of anaplastic astroglial cells. A heterogeneous cellular composition prevails with cell types such as fibrillary, undifferentiated, pleomorphic, gemistocytic, lipidized, multinucleated and granular astrocytic cells, with significant nuclear atypia and brisk mitosis (Fig. 2.2a–e). The cells are dispersed over a variably fibrillated stroma with occasional microcystic change. At times, the spindle shaped astrocytes can be arranged in interlacing fascicles imparting a sarcomatous appearance to the tumor (Fig. 2.2f). The cells may also be large with well delineated borders and at times resemble an epithelial malignancy or melanoma. This extent of cellular variety necessitates that the diagnosis of glioblastoma be based on the tissue pattern rather than on the individual cell type. Thus, the essential diagnostic features are the presence of pleomorphic anaplastic glial cells with nuclear atypia, brisk mitosis and prominent microvascular proliferation (MVP) and/or necrosis. Prominent MVP is a histopathological hallmark of glioblastoma. On light microscopy, it typically appears as glomeruloid tufts of multilayered endothelial cells which are mitotically active along with smooth muscle cells or pericytes (Fig. 2.3a). This is often found

Fig. 2.2 Heterogeneous cellular composition of glioblastoma showing fibrillary (a), undifferentiated (b), pleomorphic (c) and gemistocytic (d) morphology. Brisk mitotic activity (black arrows) is seen (e). The tumor occasionally imparts a sarcomatous appearance on histology (f) (Microscopic images a–c and f – original magnification ×80; d- original magnification ×160 and e- original magnification ×320[All H&E])
Fig. 2.3 Representative photomicrographs of glioblastoma depicting the vascular changes, necrotic pattern and the spreading front. Microvascular proliferation often has a glomeruloid appearance (a- black arrows). Necrosis can be palisading (b-black arrows) or confluent (c-white arrows), the latter accompanied by sclerosed thrombosed blood vessels (c- black arrow). Florid neovascularization is seen in most glioblastomas (d). The spreading front of glioblastoma shows characteristic peri-neuronal spread (e-black arrows), peri-vascular spread (e-white arrows) and subpial spread (e-black arrow) (Images a, d and f – original magnification ×80; b and c – original magnification ×32; e- original magnification ×160[ All H&E])

around necrosis and is directionally oriented to it (Haddad et al. 1992). Tumor necrosis is a fundamental feature of glioblastoma and may be of geographic(confluent) or palisading (historically called pseudo palisading) type (Fig. 2.3b, c). Necrosis is one of the strongest predictors of aggressive behavior among the diffuse gliomas. Another striking feature of glioblastoma is the angiogenesis (neovascularization). Glioblastoma is a highly vascular tumor and sprouting capillaries from pre-existing vessels, vessel cooption by migrating tumor cells are commonplace in glioblastoma (Fig. 2.3d). The tumor may be studded with fresh and/or old bleeds. Other salient features of glioblastoma include sclerosed and thrombosed blood vessels (Fig. 2.3c), tumor infiltrating lymphocytes and secondary structures such as satellitosis, which is the phenomenon where tumor cells line up in the sub pial/subependymal region/around neurons and blood vessels (Fig. 2.3e, f). The secondary structures, highly suggestive of infiltrating glioma, were earlier referred to as Scherer’s structures, in appreciation of the scientist whose visionary description demonstrated the most common sites for glioma invasion (Scherer and Structural 1938).

Previously, glioblastoma was called “Glioblastoma multiforme”. Though the terminology is now obsolete, it reflects on the extreme variability of the histopathology of the tumor. Some of the histological patterns observed in glioblastoma are:

1. Small cell glioblastoma (Fig. 2.4): This pattern consists of highly monomorphic small cells with round to elongated hyperchromatic nuclei with minimal cytoplasm. They tend to mimic lymphocytes and due to their uniformity, micro calcifications and chicken wire like blood vessels, they may be confused with
anaplastic oligodendroglioma (Fig. 2.4a). They may show minimal immunoreactivity to GFAP, which is a marker for astrocytes (Fig. 2.4b). However, the increased understanding of molecular signatures of gliomas has made this pattern easily discernible. The WHO 2016 has acknowledged that unlike the other
tumors that come up as differential diagnosis, small cell glioblastomas frequently have EGFR amplifications/overexpression (Fig. 2.4c, d) and chromosomal arm 10q losses. Also, 1p and 19q co-deletion which is the defining feature for anaplastic oligodendroglioma, is absent in small cell glioblastoma.

2. Glioblastoma with Oligodendroglial component (Glioblastoma-O) (Fig. 2.4e, f): This pattern had caused quite a debate while laying down the WHO 2007 classification. It was identified by C.R. Miller et al. through studies involving large cohorts of patients with mixed glioma encompassing astrocytic and oligodendroglial components and with large areas of necrosis. They found that these patients had a worse prognosis than those without necrosis (Miller et al. 2006). They had suggested that this pattern be called anaplastic oligoastrocytoma grade IV. But the majority of pathologists who convened at the WHO 2007 consensus meeting opined that more clinico-pathological data should be available before this tumour is considered a new disease entity. Thus it was decided that this pattern be termed as ‘glioblastoma with oligodendroglioma component’. Then came the histomolecular coup which has overthrown the diagnosis of oligoastrocytoma altogether in the WHO 2016 classification. With this, it is possible to classify such tumors as ‘Glioblastoma, IDH wild type’, ‘Glioblastoma, IDH mutant’ or ‘Anaplastic oligodendroglioma, IDH mutant and 1p/19q codeleted’.

3. Granular cell Glioblastoma (Fig. 2.5a): Glioblastoma sometimes consists of large cells with a granular eosinophilic cytoplasm which stains with periodic acid Schiff (PAS), usually scattered and sometimes as foci within the tumor. When granular cells predominate, the histology closely resembles that of other granular cell tumors like those arising from the pituitary stalk or other tissues. Occasionally, these cells, though more granular and larger, may be mistaken for macrophages and the lesion may be misinterpreted as macrophage-rich condition such as a demyelinating lesion, especially in the context of perivascular chronic inflammation. Such cells may be immunoreactive for macrophage markers like CD68 but not for specific markers such as CD163. GFAP may show peripheral positivity in occasional cells. Glioblastoma with this pattern has an aggressive behavior. In other diffuse gliomas too, granular cell pattern has been reported to confer poorer prognosis (Rao et al. 2017).

4. Heavily lipidized glioblastoma (Fig. 2.5b, c): Occasional glioblastomas consist of cells with foamy cytoplasm. However, rarely such cells predominate but when they do, the pattern is called heavily lipidized glioblastoma. The lipidized cells may be grossly enlarged and juxtaposed lobules of fully lipidized cells may mimic adipose tissue.

5. Glioblastoma with primitive neuronal component (Fig. 2.5d–f): This newly recognized pattern in WHO 2016 classification, was earlier referred to as ‘Glioblastoma with PNET component’ (Song et al. 2011). An otherwise classical high grade diffuse glioma with one or more foci of sharply demarcated primitive nodules showing neuronal differentiation constitutes this pattern. These foci are markedly cellular, even more than the adjacent glioma, display a high nuclear-to-cytoplasmic ratio and mitosis- karyorrhexis index and may contain variable features like Homer-Wright rosettes, cell wrapping and other features that resemble
CNS embryonal neoplasms. Similar to the primitive neuronal cells, these foci show immunoreactivity for synaptophysin, loss of GFAP expression and a high Ki-67 index. One notable feature is that though the genetic makeup is similar to glioblastoma in general, this subtype may be seen in either a de-novo glioblastoma or in a clinicopathologically defined secondary glioblastoma. However, it must be noted that be it primary or secondary, the glioblastomas with primitive neuronal component have similar survival times, with a few studies suggesting that this subset has a relatively more favourable outcome (Song et al. 2011; Joseph et al. 2013). The distinctive feature of this pattern is its high rate of cerebrospinal fluid dissemination and the frequency of MYCN or MYC gene amplification which is restricted to the primitive neuronal nodules.

Thus, there exists a great cellular pleomorphism in glioblastoma. But every histological pattern does not reflect on clinical and biological behavior of the tumor. While histopathology is quintessential for establishing diagnosis of glioblastoma, it has failed to predict the clinical behavior of the tumor based on phenotypic patterns. One classical example for this is the attempt at understanding the biological behavior of patients with glioblastoma with oligodendrogial component (He et al. 2001; Wang et al. 2012). It was previously understood that glioblastoma with oligodendrogial component had a better prognosis than classical glioblastoma (He et al. 2001; Wang et al. 2012).
2001). Nevertheless, later studies proved that this group was, in fact, a heterogeneous one with differing IDH mutational status and some of tumors previously categorized as glioblastoma-O may have in fact been aggressive anaplastic oligodendroglialomas with 1p and 19q codeletion, which is now known to be Oligodendroglialoma specific (Wang et al. 2012; Homma et al. 2006).

Hence, as explained earlier, the molecular composition, which aids greatly in the prediction of the behavior of the tumor, has been included in mainstream diagnostics. Thus, the entities of glioblastoma, as defined by the WHO 2016 are; Glioblastoma, IDH-wild type; Glioblastoma, IDH-mutant and Glioblastoma, NOS(reserved for cases where complete IDH evaluation was not done).

2.4.1 Glioblastoma, IDH-Wild Type

This is defined as a ‘high grade glioma with predominantly astrocytic differentiation; featuring nuclear atypia, cellular pleomorphism (in most cases), mitotic activity, and typically a diffuse growth pattern, as well as microvascular proliferation and/or necrosis; and which lacks mutations in the IDH genes’ (D N Louis et al. 2016).

The microscopic picture is as described above with high cellular pleomorphism, MVP and/or necrosis. In addition, the WHO 2016 classification defines this entity by the absence of IDH mutations. The immunophenotype of this entity is diverse with different variants showing different immunoreactivity. GFAP expression is usually the norm in glioblastoma. However, the degree of reactivity is markedly different among variants. Other typically expressed markers in glioblastoma are S-100, Nestin and Vimentin. Nestin may be of diagnostic use when attempting to differentiate glioblastoma from other high grade gliomas. When faced with a poorly differentiated tumor, the expression of OLIG2 may help identifying astrocytomias and oligodendroglialomas as it is not so often expressed in ependy wholemas and non-glial tumors. Classical glioblastoma sometimes also express cytokeratin AE1/AE3 or EMA. Apart from these markers, certain subsets of glioblastoma express markers specific to their genetic makeup, ex. P53 immunoreactivity in tumors with TP53 mutation leading to p53 mutant protein expression, EGFR expression in those with EGFR gene amplification, EGFR vIII expression as a result of mutation in a relatively smaller subset, H3 K27 M mutant expression, and others. Glioblastoma, IDH wild type has three variants, two of which were described in the WHO 2007 classification with a new addition in the WHO 2016 classification. The three variants identified are observed to possess a genetic profile which is more or less characteristic of the variant.

The variants are as under:

(i) Giant cell glioblastoma (Fig. 2.6a–d): This is a rare histological variant characterized by large, bizarre multi nucleated giant cells with vesicular nuclei and prominent nucleoli and an occasionally abundant reticulin network. The giant cells may contain few to >20 nuclei and occasionally contain intra nuclear
cytoplasmic inclusions. A commonly found feature of this variant is the formation of pseudo-rosette like pattern with tumor cells accumulating around the blood vessels. Unlike the classical glioblastoma, microvascular proliferation is not common in this variant. The immunophenotype of this variant is characterized by the consistent but varying level of expression of GFAP and frequent positivity for p53 mutant protein expression arising as a result of TP53 mutation (>80%). Genetically, this subset is conspicuous in its lack of EGFR amplification and homozygous CDKN2A deletion. They also frequently harbor TP53 mutations (80%) and PTEN mutations (33%). The clinical outcome of giant cell glioblastoma is somewhat better than that of classic glioblastoma.

(ii) Gliosarcoma (Fig. 2.6e–h): This variant is characterized by a biphasic tissue pattern with alternating areas displaying glial and sarcomatous components. The glial component shows typical features of glioblastoma whereas the sarcomatous component consists of bundles of spindle cells surrounded by reticulin fibres. Gliosarcoma, though usually occurs in classic glioblastomas, may also arise in ependymomas and oligodendrogliomas. A subset of gliosarcomas show additional features of mesenchymal differentiation like formation of bone, cartilage, muscle and lipomatous features. In some instances, a glioblastoma with fibroblastic proliferation due to meningeal invasion or extensive vascular sclerosis can be mistaken for a gliosarcoma. However, in gliosarcoma, significant nuclear atypia and mitosis are present in both components. GFAP stains the glial component only and not the sarcomatous component, though isolated spindle cells may be positive. The sarcomatous component variably expresses other markers like alpha-1 antitrypsin, actin and EMA. Vimentin, which is an immature glial cell marker, shows itself in both the glial as well as

Fig. 2.6 Representative microphotographs of Giant cell glioblastoma (a–d) and gliosarcoma (e–h). Giant cell glioblastoma showing several bizarre multinucleated tumor giant cells (a), with some of the giant cells containing intra-nuclear cytoplasmic inclusions (b – black arrow). Most of the giant cells are positive for GFAP (c) and show strong immunopositivity for p53 (d). Gliosarcoma shows a biphasic pattern with intermingled glial (e*) and sarcomatous component (e**). The sarcomatous component shows dense pericellular reticulin which is not seen in the glial component (f). The glial but not the sarcomatous component shows strong GFAP expression (g) and both components stain for Vimentin (h). Inset in (h) shows high MIB-1 labeling in both the components (Images a–d–original magnification × 160 and e–h–original magnification × 80 [Fig.A,B&E are H&E])
sarcomatous component. This variant is largely negative for IDH1 R132H-mutant protein. TP53 mutation is infrequent and hence p53 immunoreactivity is unusual. When positive for p53, it is identified in both glial and sarcomatous components. The occurrence of genetic alterations like TP53 mutations and IDH mutations, when present in gliosarcoma, in both glial and sarcomatous components suggest that the variant is monoclonal in origin as opposed to the earlier belief that it was polyclonal. Genetically, it differs from giant cell glioblastoma in that it contains CDKN2A deletions. EGFR amplification is infrequent in this variant, too. The clinical course of gliosarcoma differs slightly from classical glioblastoma in that it was reported in occasional cases to disseminate systemically and sometimes penetrate the skull. But the studies on the difference in outcome of gliosarcoma and glioblastoma have, so far, yielded conflicting and inconclusive results.

(iii) Epithelioid Glioblastoma: This is a newly recognized, relatively rare variant of glioblastoma (IDH wild type) is the latest addition to the glioblastoma group, added in the WHO 2016 classification. It consists of densely packed epithelioid-like cells with eosinophilic cytoplasm and paucity of cytoplasmic processes, absence of interspersed neuropil and an eccentric nucleus. Epithelioid glioblastoma is an aggressive tumor which occurs mainly in young adults and children and is associated with a particularly poor prognosis. Most commonly, it is located in the cerebrum or diencephalon (Ellison, Kleinschmidt-DeMasters, and Park 2016). BRAF V600E mutation is more common in epithelioid glioblastoma compared to other glioblastomas. VE1 antibody which recognizes V600E mutant BRAF show positivity in about 50% of these cases (Kleinschmidt-DeMasters et al. 2015). The tumor also displays retention of SMARCB1 and SMARCA4 while not expressing markers such as desmin, myoglobin, smooth muscle actin or melan A. Also, it shows immunopositivity for Vimentin and S100, expresses epithelial markers like EMA and cytokeratin and lacks IDH1 and IDH2 mutations. Copy number alterations in genes observed in adult IDH wild type glioblastomas, such as EGFR amplification and chromosome 10 losses are occasionally present (Ellison, Kleinschmidt-DeMasters, and Park 2016; Broniscer et al. 2014).

2.4.2 Glioblastoma, IDH Mutant (Fig. 2.7)

This entity is defined as a ‘high grade glioma with predominantly astrocytic differentiation; featuring nuclear atypia, cellular pleomorphism (in most cases), mitotic activity, and typically a diffuse growth pattern, as well as microvascular proliferation and/or necrosis; with a mutation in either the IDH1 or IDH2 gene’ (Ohgaki et al. 2016). Histologically, the IDH mutant glioblastomas are similar to the IDH-wild type glioblastoma, with only two significant differences. Large areas of ischemic and/or palisading necrosis are less frequent in IDH mutant glioblastoma than the IDH wild types glioblastoma (Nobusawa et al. 2009). Another difference is that focal oligodendroglioma component is more frequent in the IDH mutant glioblastoma (Lai et al. 2011).
The characteristic immunophenotype of this entity is that of IDH1-R132H mutant positivity in over 90% of cases. The remaining tumors harbor rare IDH mutations. Mutations in ATRX gene (loss of expression) are the norm rather than exception in these tumors. Also, TP53 overexpression is frequent and EGFR amplification is rare in IDH mutant glioblastomas. Another typical feature of this entity is the hypermethylator phenotype it shows. All in all, the genetic makeup of this entity confers a significantly better prognosis than the wild type glioblastoma (Table 2.3).

2.5 Molecules That Define Glioblastoma in Detail

The earliest molecules which were studied in glioblastoma are TP53 and MGMT. These were identified when attempting to understand treatment resistance of glioblastoma. Epigenetic silencing of MGMT gene through its promoter methylation resulting in better response to Temozolomide was the most advocated prognostic marker in glioblastoma for nearly a decade (Hegi et al. 2005). The twenty first century has seen a tremendous advancement of technology which opened the flood gates for molecular research and inundated the scientific world with overwhelming information on the genetic and molecular make up of various malignancies. Glioblastoma was among the earliest to be targeted by large scale molecular profiling platforms like comparative genomic hybridization (CGH), single nucleotide polymorphism (SNP) arrays and others. The Cancer Genome Atlas (TCGA), an initiative by NIH, USA, applied multiplatform profiling to systematically and comprehensively define the genomic landscape of glioblastoma (Cancer Genome Atlas Research Network 2008).
The rich data has hauled several molecules into focus which hitherto evaded the notice of researchers. This has allowed the sub classification of glioblastoma into prognostically relevant molecular subgroups. In 2006, Phillips H.S et al. have classified high grade gliomas into three subgroups- Proneural, Proliferative and mesenchymal with the proneural group showing best prognosis. They used Olig2, DLL3, BCAN(Proneural), PCNA, TOP2A(proliferative), YKL-40, CD44 and VEGF (mesenchymal) as markers to histologically identify these subtypes (Phillips et al. 2006). In the seminal paper in 2008, Parson D. W.et al. have brought to centre stage, the star molecule IDH1 (Isocitrate dehydrogenase 1) whose recurrent mutations in a small subset of glioblastoma significantly correlated with better prognosis (Parsons et al. 2008). In later years, Verhaak R.G.W et al. have revamped the molecular classification and identified 4 subgroups namely Proneural, Neural, Classical and Mesenchymal. Their study focused on alterations in PDGFRA, IDH1, EGFR, and NF1 and further highlighted the importance of IDH1 mutation which was seen in the proneural group predominantly (Verhaak et al. 2010). Though both these groups of scientists used distinct methodologies and sample sets, the proneural and mesenchymal groups were robustly concordant in their molecular profiling (Table 2.4).

In the same year, H.Noushmehr’s group identified the existence of CpG Island hypermethylator phenotype in a distinct subset of gliomas (G-CIMP). The highlights of this landmark paper are the findings that G-CIMP is tightly associated with IDH1 mutation, G-CIMP patients are younger at diagnosis and display improved survival, G-CIMP is more prevalent among low- and intermediate-grade gliomas and that G-CIMP tumors belong to the proneural subgroup.

All the high throughput studies on glioblastoma yielded an ocean of information on molecular alterations which may dictate the tumor progression. Many of these alterations were further studied and possible pathway involvement has been assessed. The subsequent sections detail each of these molecules that changed the face of glioblastoma research.

### Table 2.3 Key clinical and molecular characteristics of IDH-wildtype and IDH-mutant glioblastomas (Ohgaki et al. 2016)

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<th>IDH wild type</th>
<th>IDH-mutant</th>
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<td>Clinico-pathologically defined secondary Glioblastoma</td>
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<tr>
<td>Evolution</td>
<td>De novo</td>
<td>From lower grade astrocytoma</td>
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<td>Proportion of glioblastomas</td>
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<td>~10%</td>
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<td>Age at diagnosis</td>
<td>Usually &gt;60yrs</td>
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<td>~30 months</td>
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<td>CpG methylator phenotype</td>
<td>Less frequent</td>
<td>More frequent</td>
</tr>
<tr>
<td>EGFR amplification</td>
<td>More frequent</td>
<td>Rare</td>
</tr>
<tr>
<td>TP53 overexpression</td>
<td>Less frequent</td>
<td>More frequent</td>
</tr>
</tbody>
</table>

The rich data has hauled several molecules into focus which hitherto evaded the notice of researchers. This has allowed the sub classification of glioblastoma into prognostically relevant molecular subgroups. In 2006, Phillips H.S et al. have classified high grade gliomas into three subgroups- Proneural, Proliferative and mesenchymal with the proneural group showing best prognosis. They used Olig2, DLL3, BCAN(Proneural), PCNA, TOP2A(proliferative), YKL-40, CD44 and VEGF (mesenchymal) as markers to histologically identify these subtypes (Phillips et al. 2006). In the seminal paper in 2008, Parson D. W.et al. have brought to centre stage, the star molecule IDH1 (Isocitrate dehydrogenase 1) whose recurrent mutations in a small subset of glioblastoma significantly correlated with better prognosis (Parsons et al. 2008). In later years, Verhaak R.G.W et al. have revamped the molecular classification and identified 4 subgroups namely Proneural, Neural, Classical and Mesenchymal. Their study focused on alterations in PDGFRA, IDH1, EGFR, and NF1 and further highlighted the importance of IDH1 mutation which was seen in the proneural group predominantly (Verhaak et al. 2010). Though both these groups of scientists used distinct methodologies and sample sets, the proneural and mesenchymal groups were robustly concordant in their molecular profiling (Table 2.4).

In the same year, H.Noushmehr’s group identified the existence of CpG Island hypermethylator phenotype in a distinct subset of gliomas (G-CIMP). The highlights of this landmark paper are the findings that G-CIMP is tightly associated with IDH1 mutation, G-CIMP patients are younger at diagnosis and display improved survival, G-CIMP is more prevalent among low- and intermediate-grade gliomas and that G-CIMP tumors belong to the proneural subgroup.

All the high throughput studies on glioblastoma yielded an ocean of information on molecular alterations which may dictate the tumor progression. Many of these alterations were further studied and possible pathway involvement has been assessed. The subsequent sections detail each of these molecules that changed the face of glioblastoma research.
2.5.1 **IDH Mutations**

The discovery of IDH mutations is arguably the most significant contribution to the molecular pathobiology of glioblastoma. It has kick-started an era of genetic and molecular research and diagnosis. *IDH* enzyme is of three subtypes—*IDH1*, *IDH2*, *IDH3* (Yan et al. 2009). Five genes encode for these three subtypes. *IDH1* is predominantly cytosolic whereas *IDH2* and *IDH3* are predominantly found in the mitochondrial matrix. *IDH3* catalyses the conversion of isocitrate to α-ketoglutarate(α-KG) and NAD+ to NADH(Kreb’s cycle). The other two isoforms catalyze the same reaction, but outside of the Kreb’s cycle and reduce NADP + to nicotinamide adenine dinucleotide phosphate(NADPH). These products are essential for the generation of ATP required for the cell survival. Also, studies have shown that cells with low levels of *IDH* became more sensitive to oxidative damage. Thus, in addition to being a major enzyme in the citric acid cycle, *IDH* also plays an important role in cellular defense against oxidative stress (Marko and Weil 2013).

A multi-institutional study in 2008 by Parson et al., found point mutations in *IDH1* gene in a small subset of glioblastoma samples (Parsons et al. 2008). Further analysis showed that the IDH mutant glioblastomas corresponded pretty convincingly to the clinicopathologically defined secondary glioblastomas. This landmark finding set off further studies on grade II and grade III tumours which revealed that *IDH1* mutation is common in low grade diffuse gliomas and that *IDH2* mutation also was present occasionally. The patients with *IDH1*/*IDH2* mutated tumour had a better survival than those who did not harbour these mutations (Yan et al. 2009).

Later, when Verhaak R.G.W et al. molecularly classified glioblastoma based on gene expression, they have defined the proneural subtype as the group possessing mutations in the PDGFRA or in *IDH1/2*. The proneural GBM is further subdivided into G-CIMP positive and negative subgroups based on characteristic DNA methylation patterns that are directly linked to the IDH1/2 mutational status and better prognosis (Noushmehr et al. 2010) (Noushmehr H. et al., 2010).

*IDH1* mutations observed in gliomas are most often point mutations at position 132 (R132H) (Parsons et al. 2008), where wild type Arginine is replaced by Histidine. This mutation is also popularly referred to as canonical *IDH1* mutation. The nucleotide change causing this mutation is G395A, i.e., change of nucleotide from G to A at the position 395. Other rarer mutations at this position include R132C(Arginine to Cysteine), R132S(Serine), R132L(Leucine), R132G(Glycine), R132V(Valine). All these mutations are missense and heterozygous mutations (Table 2.5).
Table 2.5 Summary of IDH mutations and their respective nucleotide changes (Yan et al. 2009)

<table>
<thead>
<tr>
<th>IDH 1:</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Arg132His (R132H)</td>
<td>395G &gt; A</td>
</tr>
<tr>
<td>Arg132Cys (R132C)</td>
<td>394C &gt; T</td>
</tr>
<tr>
<td>Arg132Ser (R132S)</td>
<td>394C &gt; A</td>
</tr>
<tr>
<td>Arg132Gly (R132G)</td>
<td>394 C &gt; G</td>
</tr>
<tr>
<td>Arg132Leu (R132L)</td>
<td>395G &gt; T</td>
</tr>
<tr>
<td>Arg132Val (R132V)</td>
<td>394_395 CG &gt; GT</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>IDH2:</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Arg172Lys (R172K)</td>
<td>515G &gt; A</td>
</tr>
<tr>
<td>Arg172Met (R172M)</td>
<td>515G &gt; T</td>
</tr>
<tr>
<td>Arg172Trp (R172W)</td>
<td>514A &gt; T</td>
</tr>
<tr>
<td>Arg172Ser (R172S)</td>
<td>516G &gt; T</td>
</tr>
<tr>
<td>Arg172Gly (R172G)</td>
<td>514A &gt; G</td>
</tr>
</tbody>
</table>

The R132 residue is evolutionarily conserved being located in the active site of the enzyme and is essential for isocitrate binding (Xu et al. 2004). The mutation at R132 makes the protein incompatible with binding to isocitrate and abolishes its normal catalytic activity. This results in reduced levels of α-KG and NADPH, which is an important cofactor, and essential for the maintenance of normal levels of reduced glutathione (GSH) to combat reactive oxygen species. R132 mutated IDH1 has an altered binding site favouring α-Ketoglutarate (α-KG) over isocitrate which results in increased production of 2-hydroxyglutarate (2-HG), which is an oncometabolite, in the cells harbouring the mutation (Dang et al. 2010). 2-HG competitively inhibits the activity of many α-KG-dependent dioxygenases which are a diverse group of enzymes that have control over several important physiological processes like hypoxia sensing, chromatin remodeling through demethylation of histone, demethylation of hypermethylated DNA, fatty acid metabolism, and collagen modification, among others (Loenarz and Schofield 2008). Thus, IDH1 mutations lead to a series of events like DNA hypermethylation of CpG Islands in the promoters of various genes (G-CIMP), histone hypermethylation, etc. Both DNA and histone hypermethylation are thought to arrest cellular differentiation by transcriptional silencing of a broad spectrum of target genes (Turcan et al. 2012).

IDH2 is the only human protein homolog of IDH1 that uses NADP+ as a proton acceptor. What R132 is to IDH1, is what R172 is for IDH2. Five point mutations have been identified in IDH2, where arginine at 172 (R172) is replaced with glycine (R172G), methionine (R172 M), lysine (R172 K), serine (R172S), and tyrosine (R172Y).

Studies focusing on temporal sequence of genetic alterations have found that IDH1 mutations occur early in the development of IDH-mutant diffuse gliomas. This discovery saw the light of the day when Watanabe et al. performed serial biopsies from single patient and IDH 1 mutation was found to occur before the development of 1p and 19q co-deletion which lead to oligodendroglioma development and TP53 mutation which later became diffuse astrocytoma (Watanabe et al. 2009). Subsequently, ATRX mutation was found to be characteristic of diffuse astrocytomas. However, the IDH wild type gliomas, comprising predominantly the glioblastomas, are thought to develop through a separate sequence of molecular events (Fig. 2.8).
IDH mutations, due to the clinical relevance they carry, have found their way into routine diagnostics. \textit{IDH} mutational analysis was previously done by sequencing the \textit{IDH} gene. But eventually, Capper et al. have successfully raised the monoclonal antibody to mutant \textit{IDH1} R132H protein in mouse (Capper et al. 2009). This antibody is now used routinely for immunohistochemistry on formalin fixed paraffin embedded tissue. Since R132H is the most common mutation (nearly 90%), this modality of testing has gained popularity. At present, IHC for \textit{IDH1}(R132H) is performed routinely to characterize all adult diffuse gliomas (Thota et al. 2012).

Raising the antibody to IDH-1 R132H mutant protein spiked the interest of researchers across the world and immunotherapy enthusiasts went ahead and developed a vaccine against the mutant \textit{IDH1} for immunotherapy for the patients harbouring the mutation (Schumacher et al. 2014). But despite the great advancement in technology, we are still a far way behind in identifying therapeutic strategies using IDH1 mutations. Other genetic alterations that are common in IDH wild type glioblastoma are TERT promoter mutation (~80%), homologous deletion of CDKN2A/CDKN2B (~60%), loss of chromosome 10p (~50%) and 10q (~70%), EGFR alterations (mutations/ rearrangement, altered splicing and/or amplification, ~55%), PTEN mutation/deletion (~40%), TP53 mutations (25–30%), PI3K mutations (~25%) (WHO, 2016).

Glioma CpG Island Methylated Phenotype (G-CIMP), often associated with IDH mutations, frequent in the proneural group, confers better prognosis and, shows hypermethylation of CpG islands in the promoter regions of various genes. Hypermethylation is the most commonly observed epigenetic alteration resulting in silencing of the promoter regions of genes, thus altering (decreasing) gene expression. Promoters of several genes such as cyclin-dependent kinase inhibitor 2A
(CDKN2A), RB1, PTEN, TP53, MGMT, etc., have been described, of which MGMT gene promoter methylation is by far the most essential in predicting the prognosis of glioblastoma (Wick et al. 2014; Hegi et al. 2008).

2.5.2 O6-Methyl Guanine DNA Methyl Transferase (MGMT) Gene Promoter Methylation

MGMT has been extensively studied in glioblastoma. Being a DNA repair enzyme, it has attracted the attention of scientists in oncology for a very long time. Towards the end of the 20th century, study of MGMT expression and its importance in gliomas began picking up speed. Starting with protein expression studies which showed inconclusive results, the research centered on this gene in gliomas has gone a long way due to its association with response to alkylating agents, especially Temozolomide in gliomas (Hegi et al. 2005). Epigenetic silencing of MGMT gene through promoter methylation has established itself as a significant event in a subset of glioblastomas.

MGMT is the gene encoding O6-Methyl Guanine DNA Methyl Transferase which is a DNA repair enzyme which removes alkyl groups (such as methyl groups) from the O6 position of guanine within the DNA. This phenomenon is of particular importance in glioblastoma as the chemotherapeutic agent, Temozolomide, which is the current standard treatment for Glioblastoma, acts by causing damage to the tumour cell DNA by adding methyl groups to the O6 position of guanine. Such damage is usually repaired by MGMT. Thus, a tumour with high MGMT activity would be resistant to the chemotherapy. Methylation of the promoter region of this gene will silence the gene and prevent the repair of chemotherapy induced DNA damage, thus, possibly making the tumour more responsive to chemotherapy.

Due to its clinical significance, MGMT promoter methylation status has been considered in various clinical trials assessing treatment response in glioblastoma (Hegi et al. 2008; Malmström et al. 2012; Stupp et al. 2009; Stupp et al. 2014; Hart et al. 2011). The earliest clinical trials showed that a combined treatment approach (temozolomide with radiotherapy) seemed to have better outcomes with improved progression-free and overall survival compared to either treatment modality alone, raising the question of the utility of routinely testing for MGMT promoter methylation status (Hegi et al. 2005), (Stupp et al. 2009). The Nordic trial found that the patients aged over 60 years, treated with temozolomide who had tumour MGMT promoter methylation had significantly longer survival than those without MGMT promoter methylation. Standard-dose TMZ (5 out of 28 days) was found to be superior to standard radiotherapy in patients older than 70 years with methylated MGMT promoter (Malmström et al. 2012). Based on the findings of various clinical trials like NOA-8, Nordic trial, Stupp’s study, and others, the EANO suggests that the patients who are not thought to be candidates for standard chemo-radiotherapy (based on performance status), may be provided either radiation or temozolomide monotherapy, depending on their MGMT methylation status (Taylor and Schiff 2015),
Thus, testing for MGMT promoter methylation is recommended for elderly patients with glioblastoma as this could aid in the decision of the course of management. However, in younger patients, though a methylated MGMT promoter confers a good prognosis, it does not determine the choice of management, as a combined therapy is shown to be associated with better survival than monotherapy in these patients.

With increasing awareness of personalized medicine, testing for MGMT promoter methylation status is gaining impetus and is now being performed for all patients with the diagnosis of glioblastoma, if not for treatment decision, at least to understand the prognosis. MGMT gene promoter methylation testing becomes especially important in a subset of patients who develop pseudoprogression after the onset of treatment with Temozolomide. Pseudoprogression is defined by an increase in contrast-enhancement accompanied sometimes with clinical symptomatology, but there is subsequent improvement and stabilization. Its underlying mechanism could be induced by a local inflammatory reaction, with abnormal vessel permeability and edema (Hygino da Cruz et al. 2011). If an MGMT-methylated tumor under treatment with temozolomide develops pseudoprogression on imaging, chemotherapy should not therefore be stopped (Jansen et al. 2010). On the other hand, if clinical and radiological features suggestive of pseudoprogression are present in temozolomide-treated non MGMT-methylated glioblastoma, they likely represent bonafide tumor progression, and a change in therapy should be considered. More often than not, MGMT promoter methylation is seen in the patients presenting with pseudoprogression, according to a study by Stupp’s group (Weller et al. 2010).

Various methods of MGMT promoter methylation testing were assessed for accurate estimation. Immunohistochemistry was assessed for its efficiency at detecting MGMT protein expression which did not correlate significantly with promoter methylation and with survival (Christmann et al. 2010). Methylation specific PCR was made popular by Esteller M et al. which correlated significantly with survival (Esteller et al. 1999). Though various other methods like quantitative MSP (Vlassenbroeck et al. 2008), methylation specific multiplex ligation-dependent probe amplification (Kim et al. 2015) (Van den Bent et al.), combined bisulphate analysis (Mikeska et al. 2012), pyrosequencing (Christians et al. 2012) (Ronaghi et al., Christians et al.), HM27K and HM450k Bead chip (Bady et al. 2012), High Resolution melt analysis (Switzeny et al. 2016), etc. are being investigated for their efficacy, the methods being widely used clinically are semi quantitative methylation specific PCR (MS PCR) (Hegi et al. 2008), quantitative real time MS PCR(qRT-PCR) and pyrosequencing.

### 2.5.3 ATRX Mutation

The latest contribution to molecular diagnosis of gliomas is the discovery of Alpha Thalassemia/Mental Retardation Syndrome X-Linked (ATRX) gene (Wiestler et al. 2013). The gene is so named because germline mutations in ATRX are associated
with alpha thalassemia mental retardation X-linked (ATR-X) syndrome (Gibbons et al. 1995). ATRX gene is located at Xq21.1 and is a DNA helicase and chromatin remodeling protein. ATRX mutations are loss-of-function mutations. A primary function of ATRX is incorporation of histone H3.3 monomers into chromatin in collaboration with the histone chaperone protein DAXX (Death-associated protein 6) (Goldberg et al. 2010; Lewis et al. 2010). This form of chromatin remodeling is essential for maintenance of inactive proportion of the genome in a compact organization which is refractory to regulatory activity (Brennan et al. 2013). Thus, a mutation in ATRX would result in loss of this compact chromatin organization and thereby exposure of the region of genome which should be inactive.

It was in 2012 that ATRX mutations were identified in adult and paediatric gliomas (Kannan et al. 2012; Liu et al. 2012; Schwartzentruber et al. 2012). Shortly after the discovery, it had quickly gained impetus and redefined the classification of adult gliomas, as suggested by a publication from Von Deimling’s group (Wiestler et al. 2013). ATRX loss characterizes astrocytoma and is mutually exclusive with 1p and 19q codeletion which is seen in oligodendroglioma. Thus, a tumor which would have previously been thought of as a glioblastoma-O, can now be characterized using 1p and 19q codeletion status and ATRX mutational status. ATRX mutations are relatively rare in IDH wild type glioblastoma. Among the IDH mutant gliomas, about 60–70% of them show ATRX mutation (Foote et al. 2015).

ATRX loss (mutation) is strongly associated with TP53 mutation which is again, a more common feature of IDH mutant gliomas. ATRX has also been found to play a role in the regulation of telomere length as shown by studies which found that the ALT phenotype (alternative lengthening of telomeres) was significantly correlated with ATRX loss (Wiestler et al. 2013). This regulation of telomere length is essential for tumor cell immortality.

Molecular testing for ATRX is now routinely done using Immunohistochemistry (IHC). Since the mutation predominantly results in a truncated protein or abrogated protein expression, the mutant phenotype is evidenced by the loss of expression of the protein. During IHC interpretation, it is important to note that retention of ATRX expression is seen in the normal endothelial cells, native and reactive glial cells and overrun neurons. This serves as an internal control. Therefore it is important to assess ATRX immunoreactivity in the tumor core rather than its’ infiltrating front.

2.5.4 TP53 Mutation

TP53 is the most widely studied gene in cancer research. Popularly known as the guardian of the genome, its mutations have been implicated in numerous human cancers. TP53 mutation was initially thought to be the earliest mutation leading to glioma genesis, as with other cancers. It was only much later that the identification of IDH mutations being the earliest changes, even prior to TP53 mutation, surprised the world (Watanabe et al. 2009). TP53 mutations, along with IDH and ATRX mutations are now considered molecular hallmark features of diffuse and anaplastic
astrocytomas (WHO grades II and III) as well as clinicopathologically defined secondary glioblastoma (Liu et al. 2012; Gillet et al. 2014). It is also of interest to note that giant cell glioblastomas usually possess TP53 mutations (Meyer-Puttlitz et al. 1997). The cascade of molecular events triggered by the mutation of TP53 gene involving the p53/MDM2/p14ARF pathway is one of the key events in gliomagenesis.

2.5.5 TERT Promoter Mutation

Maintenance of the telomeres in the tumour cells is an essential step towards cancer cell immortality. As mentioned earlier, alternate lengthening of telomere (ALT) phenotype is associated with ATRX mutation. Another mechanism by which the tumour cells maintain the telomeres is through the telomerase reverse transcriptase (TERT) (Horn et al. 2013). It is a catalytic subunit of the enzyme telomerase. Telomerase is an enzyme which essentially maintains the telomere length. This is repressed under physiological conditions leading to progressive shortening of telomeres. The mutations leading to aberrant expression would maintain telomere length, thus imparting immortality to the cell.

TERT gene promoter mutations are point mutations usually affecting positions −228 and −250 in the promoter region, substituting a cytosine for a thymine (228 C > T, 250C > T). This unmasks a binding site for GA-binding protein (GABP) transcription factor which binds to the mutant promoter, causing aberrant expression of TERT (Koelsche et al. 2013; Arita et al. 2013).

These mutations were first discovered in melanoma, and are thought to increase the expression of telomerase, thereby maintaining telomere length and enabling repeated cell division (Horn et al. 2013). These mutations have later been identified in many CNS tumours, including glioblastoma (Killela et al. 2013). TERT mutations are more common in IDH wild type glioblastomas than in the IDH mutant form. Within the IDH wild type glioblastoma, TERT promoter mutation is inversely related to the TP53 mutations (Brennan et al. 2013; Nonoguchi et al. 2013). The IDH mutant glioblastoma preferentially makes use of ATRX mutation induced alternate lengthening of telomere pathway. TERT promoter mutations and polymorphisms have been reported to be associated with shorter survival in several studies (Mosrati et al. 2015; Spiegl-Kreinecker et al. 2015; Yuan et al. 2016).

2.5.6 Cytogenetic Abnormalities

A wide variety of chromosomal alterations are found in glioblastoma, of which, the most common alterations are gain of chromosome 7 and loss of chromosomes 9, 10 and 13. The combination of gain of 7p and loss of 10q (7p+/ 10q−) is most frequently encountered in glioblastoma (Homma et al. 2006). Chromosome 7 harbours
EGFR gene on its short arm and the key gene affected by loss of 10q is PTEN. Hence, EGFR amplification and PTEN deletion are associated with 7p+/10q-. However, mutations in these genes are less frequently encountered. Another combination of chromosomal alteration found, though less often, in glioblastoma is the combined gain of chromosomes 19 and 20.

2.5.7 **EGFR Amplification and Mutation**

As discussed above, 7p gain is associated with EGFR (Epithelial Growth Factor Receptor) gene amplification. It is more common in clinicopathologically defined primary glioblastoma and other IDH wild type gliomas and is often found in the classical glioblastomas subtype (Verhaak et al. 2010). EGFR, under normal physiological conditions, plays a central role in various normal cellular processes such as cell proliferation, differentiation and development.

EGFR is located on the short arm of the chromosome 7 (7p12) which encodes a cell surface receptor tyrosine kinase, a member of erb-1 family of receptors (Hatanpaa et al. 2010). Binding of growth factor ligand to extracellular domain and phosphorylation on the intracellular domain activates EGFR and this initiates signal transduction cascades (Ras/MAPK and PI3K /Akt) leading to increased DNA transcription, angiogenesis, anti-apoptosis and cellular proliferation.

In addition to amplification, mutations are also commonplace in glioblastoma. Several mutants may be present in one tumor itself, contributing to intra-tumor heterogeneity. EGFR mutant (EGFR vIII) is detected in about 50% of tumours with EGFR amplification. EGFR vIII is generated from a deletion in exon 2–7 of the EGFR gene which results in the frame shift deletion of 267 amino acids in the extracellular domain of EGFR (Hatanpaa et al. 2010). EGFR vIII does not bind to the growth factor ligand as the receptor is truncated with a short extracellular domain, but is constitutively phosphorylated. This structural abnormality mimics the effect of ligand binding and induces conformational change in the receptor, followed by increased intracellular signaling and cell proliferation (Nishikawa et al. 1994). Studies have shown that EGFR vIII expressing cells not only drive their own intrinsic growth but also increase the proliferation of adjacent wild type EGFR expressing cells by paracrine signaling through cytokine receptors (Inda et al. 2010).

Another mutant EGFR vII is also generated by deletion of exon 2–7 of the EGFR gene and is present in 9% of focally EGFR amplified cases. Constitutive expression of EGFR vII results in the downstream activation of Akt signaling similar to that of EGFR vIII.

In the light of molecular studies, it has become clear now that various genetic alterations involving EGFR in glioblastoma are distinct from those observed in other EGFR altered cancers. In glioma, focal EGFR amplification occurs at extremely high level. Vast majority of other mutations are EGFR vIII point and missense mutations which are found exclusively in the extracellular domain (Lee et al. 2006), while most mutations in other non-glioma cancer are found in the intracellular domain.
(Jänne et al. 2005). EGFR phosphorylation of EGFR\textsuperscript{vIII} leads to nuclear transport of EGFR\textsuperscript{vIII} and enhanced formation of a complex between EGFR\textsuperscript{vIII} and STAT3 in the nucleus suggesting that EGFR and EGFR\textsuperscript{vIII} coordinate to drive enhanced and prolonged STAT3 activity in the nucleus (Fan et al. 2013).

Though EGFR is clearly an important genetic alteration found in glioblastoma, various groups studying its association with survival and its prognostic significance have produced disagreeing results. While some groups found that EGFR \textsuperscript{vIII} overexpression along with EGFR amplification was associated with poor prognosis in younger patients (Shinojima et al. 2003), other studies showed that EGFR overexpression was associated with poor prognosis in older individuals (Srividya et al. 2010). Yet another study showed that EGFR over expression did not carry prognostic significance in the natural history of disease (Heimberger et al. 2005).

Thus identifying the EGFR gene status in glioblastoma may be useful only for identifying the subset of patients who may benefit from EGFR targeted therapy.

2.5.8  \textit{PTEN Mutation}

The chromosomal arm 10q harbours the gene \textit{PTEN} (phosphatase and tensin homolog deleted on chromosome 10), which was originally identified in 1997 as a tumour suppressor gene that was mutated in prostate, breast and brain tumours, including glioblastoma (Li et al. 1997). \textit{PTEN} protein catalyses the dephosphorylation of the 3’ phosphates of the inositol ring in PIP3, resulting in the biphosphate product PIP2. This dephosphorylation is important because it results in inhibition of the AKT signaling pathway. PI3K/AKT pathway is usually dormant in differentiated and quiescent cells. When this pathway is activated, cell cycle regulation goes hay-wire and oncogenesis ensues. \textit{PTEN} deletion primarily acts through AKT and PI3K pathway by functioning as a lipid phosphatase (Endersby and Baker 2008). Thus, \textit{PTEN} deletion and loss of 10q (including \textit{PTEN}) are associated with more aggressive phenotype (Srividya et al. 2011).

2.5.9  \textit{Platelet Derived Growth Factor Receptor Alpha}(PDGFRA)

PDGFRA makes its appearance during normal CNS development and regulates normal glial cell proliferation and oligodendrocyte differentiation (Richardson et al. 1988). PDGFRA expression has been shown to be increased in various cancers including brain tumor (Shih and Holland 2006).

The gene encodes a transmembrane protein belonging to the class III family of receptor tyrosine kinases (RTKs). The binding of ligand to this receptor triggers downstream signaling pathways like, MAPK, PI3K/AKT and JAK/STAT and plays an important role in cell proliferation, cell migration and angiogenesis (Lu et al. 2013).
2001). Thus, an enhanced expression of PDGFRA would result in excessive proliferation, angiogenesis, etc. which are features of malignancy.

In glioblastoma, amplification of the PDGFRA gene is found in 15% of all tumors, mainly in the proneural subtype of GBM (Verhaak et al. 2010). PDGFRA may be altered through various genetic mechanisms such as amplification, mutation and truncation (Phillips et al. 2013). PDGFRAΔ8, 9 is the frequent gene rearrangement in PDGFRA-amplified GBM, formed by an in-frame deletion of 243 bp in exons 8 and 9 of the extracellular portion (Kumabe et al. 1992). In addition to this deletion, in-frame gene fusion of the extracellular domain of KDR/VEGFR-2 and the intracellular domain of PDGFRA has also been found, and both of these mutant proteins were shown to be constitutively active, display transforming ability and could be inhibited using inhibitors of PDGFRA (Cancer Genome Atlas Research Network 2008). A recent study on PDGFRA amplification in a large set of pediatric and adult high grade gliomas showed that, PDGFRA amplification had no prognostic significance in pediatric high grade glioma patients but is associated with worse overall survival in adult IDH1 –R132H mutant, Glioblastoma (Phillips et al. 2013).

2.5.10 Neurofibromatosis Type 1 Gene (NF1) Inactivation

NF1 gene is a potent tumor suppressor gene which codes for neurofibromin, whose negative regulation of Ras and mTOR signaling in astrocytes is responsible for antitumor effect. Hence, an inactivation of this gene can cause tumorigenicity. The genetic alterations in NF1 gene in glioblastoma are deletions and inactivating mutations. Mutations of NF1 are predominantly seen in mesenchymal subgroup of glioblastoma (Verhaak et al. 2010). NF1 loss results in increased cell proliferation and migration that is dependent on Ras mediated hyperactivation of mTOR. Evidence from experiments using genetically engineered mouse models shows that NF1 loss in glial cells, in combination with a germline p53 mutation, results in fully penetrant malignant astrocytomas (Zhu et al. 2005), which progress to glioblastoma upon deletion of PTEN (Kwon et al. 2008).

2.5.11 Signaling Pathways Altered in Glioblastoma:

- Receptor tyrosine kinase/ PI3K/ PTEN/AKT/mTOR pathway (Altered in 88% glioblastomas (Cancer Genome Atlas Research Network 2008)) (Fig. 2.9):
- The PI3K/AKT/mTOR pathway is an intracellular signaling pathway which is important in regulating the cell cycle. Under normal physiological conditions, this pathway is essential to promote growth and proliferation over differentiation of adult stem cells and neural stem cells specifically (Peltier et al. 2007). The first intracellular component of this pathway is phosphatidylinositol 3-kinase (PI3K)
complex, which, when activated sets into action a series of genes with Akt (also called protein kinase B) first, followed by mTOR, which integrates several upstream signals into effector actions on multiple downstream targets involved in cell growth and division (Mao et al. 2012). The triggering stimulus for the cascade of events is the activation of receptor tyrosine kinase family members, most notably, EGFR, ERBB2, PDGFRA, c-MET, etc. Hence, gene activating alterations like gene amplification or activating mutation will lead to the cascade of events which enhance cell proliferation. However, genes like PTEN usually put a check on cell proliferation by inhibiting PI3K. Thus, an inactivating mutation or deletion of this gene will not limit the activation of PI3K, leading to aberrant cell proliferation (Cancer Genome Atlas Research Network 2008).

- **P53/MDM2/p14ARF pathway** (altered in 87% glioblastomas) (Cancer Genome Atlas Research Network 2008) (Fig. 2.9): P53, as described earlier, is a key tumor suppressor gene and also a broad transcription factor which regulates over 2500 genes involved in tumorigenesis and tumor invasion. An inactivating mutation in TP53 or negative regulation of TP53 results in tumorigenesis. MDM2 and MDM4 are essential negative regulators of TP53 gene. MDM2 may inactivate p53 through transcriptional inhibition by direct binding, and degradation through its E3 ligase activity. MDM4 inactivates p53 only through transcriptional inhibition. Thus, gene activating alterations in MDM2 and MDM4 such as amplifications inhibit p53 and contribute to oncogenesis. A gene CDKN2A (p14 ARF), which is further upstream to MDM2, inhibits regulation of p53 pathway by directly binding to MDM2 and subsequently inhibiting its E3 ubiquitin ligase activity (Toledo and Wahl 2007) (Kamijo et al. 1998). Thus, an inactivating alteration in CDKN2A results in uninhibited action of MDM2 which in turn, inhibits p53. This pathway is altered in 87% of glioblastomas.
CDKN2A/CDK4/retinoblastoma protein pathway (altered in 78% glioblastomas) (Cancer Genome Atlas Research Network 2008)) (Fig. 2.10):

The RB gene codes for a tumor suppressor protein retinoblastoma (pRB) which plays a crucial role in inhibiting cell cycle progression by binding to and inhibiting transcription factors of the E2F family. Hence, an inactivating alteration of RB gene leads to uninhibited cell division. The RB gene is negatively regulated by the complex of cyclin-dependent kinases (CDKs), notably, CDK4, CCND2, CDK6. Thus, an activating alteration in these kinases accentuates the inhibition of RB gene, leading to excessive cell proliferation. These Cyclin Dependent kinases are normally inhibited by CDK inhibitors, CDKN2A, CDKN2B and CDKN2C. When these inhibitors suffer inactivating alterations, it would result in unchecked cyclin-dependent kinase activity and thus, exaggerated RB inhibition, thus leading to aberrant cell proliferation (Cancer Genome Atlas Research Network 2008; Mao et al. 2012).

2.6 Molecular Biology of Recurrence in Glioblastoma Tumors

Glioblastoma is notorious for its inevitable recurrence after maximal safe resection despite concomitant radiation and chemotherapy following surgery. The recurrent tumor tends to come back with a vengeance and is more resistant to therapy. Currently, there is no accepted standard therapy for recurrent glioblastoma. A select
few patients derive benefit from a re-surgery with majority being left out in the pro-
verbial cold due to lack of approved therapy with promising results. Strikingly, not
many studies focus on recurrent glioblastoma. One major reason for this is that not
all recurrent tumors are operable, limiting the access to the recurrent tumor tissue.
Thus, paired tumor sample scarcity precludes any molecular studies on recurrent
glioblastoma. As a result, research on recurrence has been mostly limited to docu-
mentation of clinical characteristics and very few clinical trials using angiogenesis
inhibitors, etc. Our knowledge on recurrence mainly stems from a handful of studies.
Recurrences are predominantly local (recurring within 2 cm margin of the original
tumor) with only a small proportion coming back as distant recurrences(recurring
distantly in a different lobe or in contralateral hemisphere). The genetic makeup of
the local and distant recurrences when compared to their primary counterparts still
remains largely unknown, though some recent studies have attempted to answer this
question. The genetic landscape of local recurrences was thought be similar to the
original tumor and the distant recurrences were argued to be possibly second primary
tumors (Reis et al. 2001; Martinez et al. 2003). However, other researchers identified
sufficient similarities between the primary and distant recurrent tumors to conclude
that they were indeed remote recurrences rather than entirely new primary tumors
(van Nifterik et al. 2006). In a recent study comparing the mutations in the local and
distant recurrences with their original primary tumors, distant recurrences were
found to share an average 25% of mutations with their primary tumors while local
recurrences possessed an average of 70% of shared mutations (Kim et al. 2015).

The few studies which attempted to understand molecular profile of recurrent vs
primary tumors, had compared only a few candidate genes and had a small sample
size and none of them considered the intratumor heterogeneity (Campos et al.
2016). A summary of the studies is listed in Table 2.6.

Mounting evidence points towards definite alterations in recurrent glioblastoma,
the nature of which depends on the profile of first tumor. For example, in conjunc-
tion with the above studies, other researchers have found evidence of tumor
evolution possibly in response to radio-chemotherapy. For example, primary glio-
blastomas with MGMT promoter methylation were found to lose the methylation
with higher MGMT expression in recurrent tumors (Christmann et al. 2010).
Consequently, a series of clinical trials with dose-intensified treatment with
Temozolomide came into effect, however, the results were not promising (Gilbert
et al. 2013). Kim, et al., while analyzing 21 paired samples, found that one recurrent
glioblastoma had a hypermutated phenotype and that was originally an IDH1
mutant. The authors suggested that IDH1, commonly associated with a hypermeth-
ylator phenotype may have suppressed MGMT and rendered the tumor more sus-
ceptible to temozolomide induced mutagenesis. Interesting to note is the fact that
majority of the accumulated mutations were found to affect mismatch repair genes
like PMS1 and MSH5 (Kim et al. 2015).

Evidence from several studies which assessed the possible effect of treatment
modality on glioblastoma recurrence suggests that, altogether, glioblastomas
undergo evolutionary change and selective pressures such as radio-chemotherapy
and targeted therapies are likely to alter the molecular composition of these tumors
Table 2.6  Summary of genetic alterations studied so far in recurrent glioblastoma

<table>
<thead>
<tr>
<th>Genes studied</th>
<th>Findings</th>
</tr>
</thead>
<tbody>
<tr>
<td>MLH1, MSH2, MSH6</td>
<td>Expression lower in recurrence (Stark et al. 2010; Shinsato et al. 2013)</td>
</tr>
<tr>
<td>TP53 and PTEN mutation, EGFR amplification</td>
<td>One study found lower expression at recurrence (Stark et al. 2003). Another study identified two distinct patterns of accumulation of molecular alterations depending on the profile of the original tumor (Martinez et al. 2010).</td>
</tr>
<tr>
<td>Methylation of promoters of MGMT, CASP8, CASP3, CASP9, DCR1, DR4, DR5, TMS1, CDH1, CDH13, RASSF1A, BLU, CHFR, CASP8</td>
<td>More methylation observed at recurrence (Martinez et al. 2007)</td>
</tr>
<tr>
<td>miRNA-10b, miRNA-21, miRNA-181b, miRNA-181c, miRNA-195, miRNA-221, miRNA-222</td>
<td>No change observed at recurrence (Ilhan-Mutlu et al. 2013)</td>
</tr>
</tbody>
</table>

(Campos et al. 2016). However, the original tumor composition plays a significant role, of which, tumor heterogeneity is a major player. As discussed earlier, glioblastomas possess such heterogeneity as not seen in most other malignancies. Intertumor heterogeneity is clearly evidenced by histomorphology. But intratumor heterogeneity of glioblastoma is unmistakable when molecular profiling is done. For instance, Andor et al. have found as many as seven subclones within 100 mg of tumor tissue (Andor et al. 2014). This heterogeneity makes it difficult to predict which clonal subtype has re-emerged and hence, will respond to which type of agent. Though angiogenesis inhibitors like Bevacizumab have seen response in some patients, intratumor heterogeneity clearly explains its failure in other patients. The same group studied the effect of Temozolomide on the number of subclones in recurrent tumors, and showed that three types of changes may occur in this respect. A dominant subclone in primary tumor may shrink and disappear in recurrence or a dominant subclone may resist treatment and remain a dominant subclone or a smaller subclone may resist treatment and emerge as the dominant subclone in recurrence. Thus, future research focusing on understanding intratumor heterogeneity and predicting the possible molecular landscape of recurrent glioblastoma will greatly help in decision making in the treatment of recurrent glioblastoma.

Another line of research in recurrence has focused on the cancer stem cells present within the tumor which are relatively slow-dividing as compared to the fast-dividing glioblastoma cells (Lathia et al. 2011; Richichi et al. 2013). It is thought that through various cell to cell signaling methods, the therapy resistant, slow-growing cells are maintained in a quiescent state by the fast-growing neighboring cells. When these inhibitory signals are lost due to resection of the tumor, the remaining slow-growing cells regain their proliferative potential to cause recurrence. Cancer stem cells are resistant to therapy due to various properties analogous to normal stem cells, like overexpression of DNA-damage repair enzymes (Bao et al. 2006), metabolic traits which allow for their growth in hypoxic conditions (Li et al. 2009), their slow growth helping them escape the routine therapy which is...
targeted at proliferating cells, etc. Therefore, glioblastoma cancer stem cells play a role in recurrence.

Further research attempting to identify molecular patterns of recurrence in glioblastoma is direly needed to address the pressing issue of tumor recurrence and consequently poor survival in glioblastoma patients.

### 2.7 Summary

In view of the evolving molecular landscape of glioblastoma and the emphasis on arriving at a histomolecular diagnosis, one must bear in mind the following essentials:

(a) Two entities of Glioblastoma are now recognized- IDH wild type and IDH mutant.

(b) Clinicopathologically defined primary (de novo) glioblastoma is usually IDH wild type and clinicopathologically defined secondary glioblastoma corresponds to IDH mutant type.

(c) IDH mutant type has a significantly better prognosis than an IDH wild type glioblastoma.

(d) MGMT promoter methylation confers better prognosis to the patients with glioblastoma and is an independent prognostic factor.

(e) Pseudoprogression is commonly associated with MGMT methylated phenotype and resolves with steroids and Temozolomide therapy is continued.

(f) In MGMT unmethylated cases, pseudoprogression is usually rare and it is more likely to be recurrence rather than pseudoprogression. They are less likely to respond to Temozolomide therapy and other drugs may be required.

(g) Molecular sub-classification of glioblastoma may be of prognostic value with proneural type showing better prognosis which may, again be due to the fact that IDH mutations occur with high frequency in the proneural type.

(h) Most common molecular alterations conferring poor prognosis in glioblastoma are: EGFR amplification with EGFRvIII mutation, PTEN deletion, TERT promoter mutation.

### 2.8 Clinical Trials

Clinical trials using targeted drug therapies against IDH1, EGFRvIII, Tyrosine receptor kinases, etc., have been performed with mixed results. IDH1 R132H vaccine has been developed in the hope that they can be of use in IDH mutant glioblastomas (Schumacher et al. 2014). It has, so far, shown promising results in animal models (Dimitrov et al. 2015). Two clinical trials are currently in effect using IDH 1 and 2 mutant inhibitors.
Another molecular alteration that has successfully reached the clinical trial phase is the EGFR vIII mutant amplification (Taylor et al. 2012). Animal experiments have shown that tumors with this mutation are found to be sensitive to Cetuximab (Padfield et al. 2015). Several small molecule inhibitors, vaccines developed against various mutants have failed to show significance in the treatment of glioblastoma, the possible reason could be the extensive intratumor heterogeneity seen in glioblastoma.

2.9 Need of the Hour

As discussed above, the various exploratory studies carried out in glioblastoma have unearthed several mutations, epigenetic modifications, chromosomal aberrations, gene copy number changes, etc. But we are still a long way from developing a therapeutic strategy which will increase the longevity of the patients with good quality of life. The first hurdle towards achieving this is the incomplete understanding of the pathobiology of glioblastoma. Though the large scale ‘omic’ studies have made significant contribution towards this, there remain various missing links in the pathways and the holistic picture is lacking. The key areas of research currently in vogue in glioblastoma are aimed at understanding inter and intratumor heterogeneity, glioblastoma cancer stem cell biology, angiogenesis, tumor metabolism, resistance to therapeutic response, etc. However, a major aspect of glioblastoma, which is its invariable recurrence, has been relatively less studied and the treatment of patients with recurrence poses a dilemma to the treating clinicians. Limited number of hypotheses trying to explain the inevitable recurrence have been put forth, of which, the theory in vogue is that since the tumor cannot be completely resected due to its diffuse nature, tumor cells which escape resection may also resist radiation and chemotherapy and cause recurrence. Further research addressing this question is essential to help those patients who may have benefited from the primary treatment but presented with recurrence.

The initial excitement generated due to revelation of molecular landscape of glioblastoma had kick started the development of several inhibitors, vaccines, etc. for targeted therapy. Several of these molecules had passed the test in vitro and in animal models but failed to show significant results in the next phases. But the lesson learnt from this exercise is that the need for personalized treatment is paramount in glioblastoma due to its vastly varied features. Inter and intra tumor heterogeneity has to be fully understood if the hope for development of therapeutic strategies for glioblastoma is to be realized.

Though the data is plentiful and more is being generated this moment, there is need for further more fundamental research to understand the biological behavior of this tumor, which is the only way to handpick the right molecules which could serve as drug targets. The meaningful interpretation of the enormous data, prospective studies in the clinical setting and functional studies on the bench, building a bridge between the bedside and the bench are the need of the hour. After all, the end point to any disease related research is the benefit of the patient.
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