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Epigenetics of Glioblastoma Multiforme

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Abstract

Aberrations in the epigenetic machinery of the genome may results the inactivation of critical genes and the epigenetic changes are important mechanisms in the evolution of malignancies that not only contributes to tumorigenesis but may also precede genetic changes. Several epigenetic mechanisms have been observed in glioblastomas including DNA hyper-methylation of genes, histone modifications including methylation and acetylation, nucleosomal rearrangement and dysregulation of noncoding RNA expression have been shown to play a critical role in the biology of glioblastomas and to contribute to the clinical outcome. This review examines the general role of epigenetic changes in the malignant process and focuses on the known epigenetic changes and development of new therapeutic strategies against these malignancies.

Keywords: Glioblastoma; Epigenetics; Methylation

Introduction

Glioblastoma multiforme (GBM) is the most malignant forms of astrocytoma [1,2]. It is the most common primary brain tumour in adults, accounting for 80% of all high-grade primary central nervous system (CNS) neoplasms [3,4]. GBM can be sub-classified into multiple groups, indistinguishable by histological appearance, but correlating with molecular-genetic factors as well as key clinical variables such as patient age and tumour location. The gliomagenesis is multistep process and the combination of acquired genetic abnormalities plays a crucial role in the progression of the tumour. The glioblastoma has been preceded by a number of recognizable lesions and the timing of these changes has been documented to be associated with the occurrence of abnormalities in different tumour suppressor genes.

Median survival of GBM is approximately 12 to 14 months and the prognosis and survival of patients remains poor [1,3,4]. The current treatments of these tumours are surgical resection, irradiation and conventional chemotherapy generally inadequate and unable to overcome the malignant biology of the tumour [5,6]. The currently used drugs are nonspecific and non-targeted nature (e.g., alkylating agents, DNA topoisomerase inhibitors, spindle poisons) [7,8] so the result of conventional chemotherapy is poor. The conventional forms of treatment for GBM are not predicated on the biology of the malignant phenotype. Due to the insufficiency of conventional therapeutic approaches for GBM, new treatment modalities must be developed that have a more molecular, "targeted" mechanism of action [9-14].

The genome of glioblastoma cells shows global hypo-methylation with specific areas of hypermethylation. Hypermethylation mostly occurs at the promoter CpG islands of genes that are associated with tumour suppression. This pattern has been associated with increased genetic instability, silencing of tumour suppressor genes such as TP53 and PTEN, and activation of oncogenes. The Cancer Genome Atlas project has identified a glioma CpG island methylation phenotype that correlated with younger age, a proneural gene expression profile, and longer overall survival in glioblastoma patients [15].

The remarkable advances in defining the GBM cell of origin have been paralleled by insights into the genetic and epigenetic underpinnings of this disease.

EGFR, PDGFR, PI3K, NF1, TP53, Rb, IDH1/IDH2 and FGFR are only a few GBM mutational "drivers," these are important mutations to understand the genomic networks misregulated in GBM. The Cancer Genome Atlas (TCGA) Research Network proposed "proneural" "mesenchymal" "classical" and "neural" as a four subtypes of GBM based on genomic profiling of hundreds of human samples. Proneural GBMs show altered expression of PDGFRA, IDH1, TP53, PTEN mutation and CDKN2A loss. Mesenchymal GBMs have deletion of NF1, mutation of TP53 and PTEN, and loss of CDKN2A. Classical GBMs carried EGFR amplification and lack of PTEN, and CDKN2A and the neural GBMs show a strong expression of neuron markers and genes associated with neuron projection and axon and synaptic transmission.

microRNAs and long non-coding RNA are affect gene expression through regulation of mRNA stability and transcription regulation. MicroRNAs are non-coding RNAs, which bind to microRNA response elements (MREs) in target mRNAs. miRNA is bind to the RISC complex (RNA-induced silencing complex), the miRNA/RISC complex binds the target mRNA, thereby modulating its stability. miRNAs dysregulated in glioma include miR10b, which is expressed in glioma tumours and stem cells and miR10b controls GBM cell and stem cell cycle traverse and is correlated with poor prognosis (http://tcga-data.nci.nih. gov/tcga) [16].

Long non-coding RNAs (lncRNAs), which are control global gene repression. lncRNAs control multiple tumour suppressor proteins, oncogenes and modulate transcription, regulate post-transcriptional RNA processing, influence translation, alter DNA methylation and chromatin architecture through local (cis) and long distance (trans) mechanisms. lncRNAs may play a crucial role in GBM development and progression. lncRNA MEG3 has been implicated in glioma cell

proliferation and MEG3 expression is associated with differential methylation[16].

Genetic changes are well recognized in GBM and include alterations in the Rb/p16 pathway (>90%) loss of heterozygosity of 10q (70%), EGFR amplification (34%) and TP53 mutations (31%) [17]. Epigenetic changes, also known to occur in GBM, are less well characterized and their relation to clinical outcome is as yet uncertain.

There are several different components for genetic and epigenetic alterations that may be required for driving the unmitigated proliferation of glioblastoma cells.

Epigenetic Alterations and Glioblastoma Progression

Cancer progression is the current concept of cancer epigenetics. Epigenetics is the mitotically heritable changes in gene expression that are not due to changes in the DNA sequences. One of the best characterized epigenetic markers is DNA methylation. The initial finding of global hypo-methylation/hypermethylation in tumours [18] and the more recent discovery of induced inactivation of microRNA genes [18-20].

The DNA methylation occurs in cytosines that precede the guanines and these are commonly called dinucleotide CpGs. CpGs are not randomly distributed in the genome, but instead are present as CpG-rich regions referred to as CpG islands, which occur at the 5' end of the genes. These islands are usually not methylated in the normal tissues. DNA methylation occurs due to the chemical modifications of the histone proteins. Histones not only package DNA but also act as regulators of gene expression.

Locus-specific hypermethylation is frequent in GBM. The hypermethylation status of tumour suppressor genes is contributed to the pathogenesis of glioblastoma. In GBM, CpG island promoter hypermethylation occurs at genes to tumorigenesis and tumour progression, including tumour suppressors (RB1, EMP3, RASSF1A and BLU) [21], cell cycle regulation (p16INK4a and p15INK4b), DNA repair (MGMT, MLH1), apoptosis (DAPK, TIMP3 and CDH1), angiogenesis, invasion and drug resistance [22]. Patterns of DNA methylation in primary GBM specimens have been catalogued and include disruptions at many novel candidate tumour suppressors, such as the cell motility regulator *TES* (testis-derived transcript) as well as many polycomb repressor complex 2 (PRC2) target genes [23].

Hypermethylation of the tumour suppressor Rb [24] was soon followed by the discovery that this mechanism also contributed to the suppression of other genes such as VHL [25], p16INK4a [24], and BRCA1 (breast cancer susceptibility gene 1) [26].

Increased expression of EZH2 correlates with poor prognosis in GBM. The EZH2 genetic inhibition or pharmacologic inhibition can prevent self-renewal and tumourigenicity of glioblastoma cancer stem cells [21]. Caren et al was shown that the BMPR1B down-regulated in about 20% of primary glioblastoma tumours, and this is correlated with increased promoter DNA methylation [21].

The hypermethylation of genes might act as a predictor to treatment response and the MGMT (O6-methylguanine DNA methyltransferase) is the most studied DNA repair gene for the methylation-associated suppression of the DNA repair protein in glioblastomas [27]. MGMT essentially reverses addition of alkyl groups to the guanine base of the DNA making it prone to attack by alkylating agents [28]. Two studies showing that the hypermethylation of MGMT is an independent predictor for favorable prognosis in glioblastomas treated with either carmustine [29] or temozolomide [30].

Methylation of MGMT promoter was a correlative marker for response to alkylating agents; a recent clinical trial by Stupp et al. was established a new standard using chemoradiation with concurrent and adjuvant temozolomide for treatment of patients with newly diagnosed GBM, and the methylation status of MGMT promoter was correlated it with outcome [31].

Hegi et al. showed that methylated MGMT patients had an improved survival after chemoradiation than those with unmethylated MGMT, suggesting a direct predictive relationship between MGMT function and sensitivity to alkylating agents [30]. If improved outcome is seen in MGMT promoter methylated patients but who do not receive treatment with alkylating agents, this result suggest that a mechanism independent of MGMT-mediated resistance to alkylating agents could be responsible for this effect. Recent studies were showed MGMT methylation is not predictive marker in primary GBMs without any treatment applied patients [32].

Sato et al demonstrated that in stem-like glioblastoma cells the MGMT expression contributes to temozolomide resistance and a signalling pathway, MEK-ERK-MDM2-p53-MGMT, is operative in stem-like glioblastoma cells and plays a key role in the regulation of their MGMT expression. The temozolomide treatment combination with MEK targeting is effectively reduces tumorigenic potential of stemlike glioblastoma cells. This combination could also contribute to prevention of tumour recurrence [33].

Oberstadt et al showed significantly increased MGMT and ABCB1 promoter methylation in GBM tissues, but no significant association between overall survival of glioblastoma patients and MGMT or ABCB1 promoter methylation [34].

PTEN is the another critical tumour suppressor gene, the protein product for the PTEN is the functions as an inhibitor of PI3-kinase and inhibits the PI3K-Akt survival pathway, is associated with de novo GBM and less commonly with secondary GBM [35]. PTEN gene is inactivated by mutation in 20–40% of these tumours; loss of PTEN expression can occur in the absence of mutations, suggesting an epigenetic means of silencing. Baeza et al. reported PTEN promoter methylation in 35% (22/77 tumors) of primary GBM [36]. Weincke et al. reported methylation of a region approximately 2.4 kb upstream of the start site of PTEN which was frequently seen in secondary GBM but not in de novo GBM [37]. This finding was seen in low-grade astrocytoma and suggesting that PTEN promoter methylation was an early event in the evolution of these tumors progression to glioblastoma.

Hypermethylation of TMS1/ASC (target of methylation-induced silencing-1), an adaptor protein that transduces death receptor apoptosis signals and has a role in inflammation, has been reported in 21% of GBM by Martinez et al.; the particular significance is the observation that 57% of long-term survivors (>3 years) had promoter methylation of TMS1/ASC [38].

Stone et al. also reported TMS1/ASC promoter methylation in 43% of GBM but did not report association with survival [39]. Folz et al. were identified BEX1 and BEX2 as tumour suppressor genes, the promoters of which are hypermethylated in GBM but not in non-tumor brain tissue and demonstrated that overexpression of these genes in glioma xenografts suppressed tumor growth [40].

Knobbe et al. reported epigenetic silencing of carboxyl-terminal modulator protein (CTMP) gene, the products of the CTMP acts as a negative regulator of Akt, in 40% of GBMs tested, and the aberrant expression of CTMP in dysregulation of survival pathways in GBM [41]. The SLIT gene family is important in migration and axonal guidance and the the epigenetic inactivation of this genes were reported by Dallol et al. the promoter hypermethylation of the SLIT2 in 59% of glioma tissue samples tested, whereas in non-tumour brain tissue, the promoter was fully unmethylated [42]. This did not correlate with clinical outcome, but in a subset of tumours, the methylated tumours also had lower protein expression.

Watanabe et al. reported promoter methylation of the P73 in 5 of 28 (18%) GBM patients but not in low-grade or anaplastic astrocytoma [43].

The analysis of the methylation status of cytokine signalling (SOCS)1-2-3 in GBMs showed methylation of SOCS3 promoter to be significantly associated with an unfavorable clinical outcome [44]. Zhou et al showed SOCS1, but not SOCS3 that was epigenetically silenced in GBM [45]. The N-myc downstreamregulated gene 2 (NDRG2) is a repressor of tumour cell proliferation, was found %62 hypermethyleted in primary GBM, but not in secondary or lower grade astrocytomas [46].

Schwatzentruber et al. showed that somatic mutations in the H3.3-ATRX-DAXX chromatin-remodelling pathway frequently occur in paediatric GBMs and are associated with alternative lengthening of telomeres and genomic instability [47]. Another approach to discovery is to consider the epigenetic drivers of gliomagenesis. Strum et al. incorporated the mutational status of H3F3A and IDH1 with differences in global methylation patterns in GBMs to identify 6 distinct epigenetic subgroups, which correlate with distinct clinical characteristics [48].

Doxorubicin (DOX) is a chemotherapeutic drug for cancer treatment. DOX acts as a topoisomerase I poison by preventing DNA replication and also DOX can be involved in epigenetic regulation of gene transcription through downregulation of DNA methyltransferase 1 (DNMT1) then reactivation of DNA methylation-silenced tumour suppressor genes in glioblastoma (GBM) [49].

Glioma pathogenesis-related protein 1 (RTVP-1) has oncogenic features in glioblastoma (GBM; World Health Organization class IV) and highly expressed in GBMs [50]. The RTVP-1 promoter was hypomethylated in GBM. Hypermethylation of the RTVP-1 promoter was associated with improved overall survival in GBMs and the overexpression of this gene was associated with increased proliferation, enhanced invasion and inhibition of apoptosis [51,52].

microRNA-137 (mir-137) was described as a regulator of RTVP-1 gene. Downregulation of mir-137 contributes to the high expression of RTVP-1 in glioblastoma. The current study describes further loss of regulatory control of RTVP-1 in GBM by promoter methylation. Unlike GBM, RTVP-1 was hypermethylated in oligodendroglioma, another astrocytic tumor. While this may indicate specificity of RTVP-1 hypomethylation in GBM, it may also reflect general hypermethylation observed in oligodendrogliomas [53-55].

Besides inactivation of DNA repair genes, DNA methylation analyses showed that silencing of negative regulators of mitogenic pathways or activators of apoptosis is common in cancer showing tumor type-specific patterns. In GBM the WNT pathway may be activated through promoter methylation of negative regulators such as the WNT inhibitory factor 1, the family of secreted frizzled-related proteins (sFRPs), dickkopf (DKK), and naked (NKDs) [56,57]. Another example is the Ras pathway that in a subset of GBM is deregulated by silencing of the negative regulators Ras association (RalGDS/AF-6) domain family members RASSF1A and RASSF10 [58,59].

GBMs showed low levels of NPTX2 transcripts and the overexpression of NPTX2 gene induced apoptosis, inhibited proliferation and anchorage-independent growth, and rendered glioma cells chemosensitive. Furthermore, NPTX2 repressed NF-κB activity by inhibiting AKT through a p53-PTEN-dependent pathway, thus explaining the hypermethylation and downregulation of NPTX2 in NF-κB-activated high-risk GBMs. Prosurvival NF-κB pathway activation characterized high-risk patients with poor prognosis, indicating it to be a therapeutic target [60].

Global hypomethylation occurs at a high frequency (80%) in primary glioblastomas and the level of hypomethylation varies between GBMs, ranging from near normal brain levels to approximately 50% of normal [22].

Somatic Mutations Causes DNA Methylation

Promoter-associated CpG islands DNA hypermethylation of tumour suppressor and DNA repair genes, has been the most studied epigenetic alteration in human neoplasia [61,62]. Improvement of technology in the last few years allows comprehensive analysis of genome-wideDNA methylation on high-throughput platforms. Large-scale analysis in GBM on aberrant DNA methylation at CpG sites has unraveled a plethora of genes that are affected [55]. Widespread CpG island promoter methylation, also referred to as the CpG island methylator phenotype (CIMP), was first identified by Toyata et al [63] and has been extensively studied in many cancer types. The first two studies by Noushmehr et al [55] and Turcan et al [64] showed that glioblastomas with a hypermethylator phenotype are associated with somatic mutations in isocitrate dehydrogenase-1 [55,64] and that somatic mutations in IDH1, IDH2, as well as loss-of-function mutations in ten-eleven translocation (TET)-methylcytosine dioxygenase-2 (TET2) establish a hypermethylation phenotype in leukemia [65]. As we know about that the glioblastomas have a two distinct subtypes which are primary and secondary GBM. These are the two distinct disease entities which are the genetic and the epigenetic background of these tumors are highly variable. From a clinical and biological perspective, two main subtypes of this malignancy can be distinguished: primary (or de novo) glioblastoma develops without the presence of any precursor neoplastic lesion and manifests after a short clinicalhistory; secondary glioblastoma develops from lower grade tumors [66]. Mutations of IDH1 are rare in primary GBM (10%) and frequent in secondary GBM (80%) [67]. Than the primary glioblastomas which are showed low frequencies of IDH1 mutations and G-CIMP have a different pathogenetic/epigenetic origin than secondary glioblastoma and should be classified separately. The correlation of the neomorphic IDH1/2 mutants with a DNA methylator phenotype was also observed in acute myeloid leukemia (AML). This provided an important mechanistic link, together with the fact that IDH1/2 mutations in leukemia were exclusive with tet oncogene family member 2 (TET2) mutations [65,68]. Moreover, a high frequency of IDH1 mutations indicates a link between metabolic alterations and epigenetic modification in these tumors [15]. A mutation in IDH1 inhibits DNA demethylation and causes accumulation of the methylated DNA. IDH are NAD+ and NADP+-

dependent enzymes that catalyze the tricarboxylic acid (TCA) cycle, and mutations in IDH1 produce a metabolite called 2-hydroxyglutarate (2-HG) to accumulate. The accumulated 2-HG impairs the activity of ten-eleven translocation (TET) methylcytosine dioxygenase, which results in DNA hypermethylation. IDH1 mutation may result in G-CIMP through inhibition of the TET-mediated production of 5-hydroxymethylcytosine (5hmC), which is a primary mode of DNA demethylation [69].

Exome sequencing studies showed that at least one somatic mutation in genes which are DNA methylation (isocitrate dehydrogenase [IDH] 1, IDH2), histone modification (mixed lineage leukaemia 2 [MLL2], MLL3, MLL4, Enhancer of zeste 2 [EZH2] and histone deacetylase 2 [HDAC2]) and chromatin remodelling (a-thalassaemia/mental retardation syndrome X-linked [ATRX], death-domain associated protein [DAXX], CREB binding protein [CREBBP] and SWI/SNF-related matrix-associated, actin-dependent regulator of chromatin A2 [SMARCA2]) associated with chromatin modification [70]. These mutations result in the impairment of DNA methylation, histone modification and nucleosome positioning, and are associated with aberrant gene expression [71,55]. The crosstalk between the genome and epigenome might suggest new molecular targets and possibilities for the treatment of GBM [69].

Methylation of K27 and K36 is also disrupted by elevated levels of the onco-metabolite 2-hydroxyglutarate (2-HG) resulting from gain-of-function mutations in *IDH1* [68,72], Which was previously shown to be associated with a distinct Glioma-CpG-Island Methylator Phenotype (G-CIMP) [55].

Are Epigenetic-based therapies possible for GBM?

Epigenetic modifications by its nature are reversible; therefore changes in the epigenome associated with cancer are potentially reversible. It opens up the possibility of using epigenetic drugs which may have a powerful impact on various cancers, including glioblastomas and the major unresolved problem for epigenetic therapy of cancer is target specificity [22]. Two demethylating agents, i.e., inhibitors of DNA methyltransferase (5-azacytidin and decitabin) were approved by the Food and Drug Administration (FDA) in the treatment of myelodysplastic syndrome and a few inhibitors of histone acetylases (vorinostat, romidepsin and panobinostat) are approved in the treatment of hematological malignancies, particularly in refractory or relapsed cutaneous T-cell lymphoma [73]. Other compounds are presently in clinical trials and the hypomethylating agents are also one of the few epigenetic therapies that have gained FDA approval for routine clinical use. Small-molecule inhibitors of histone demethylases are at various stages of development and emerging preclinical data show the therapeutic potential of compounds [74].

The DNMT inhibitor Decitabine (5-aza-2'-deoxycytidine) and the HDACi suberoylanilide hydroxamic acid (SAHA; Vorinostat) are the epigenetic-based therapies and only HDACi are in trials for GBM [22]. HDACi are hydroxymates (SAHA, TSA), cyclic peptides (depsipeptide), aliphatic acids (valproic acid, butyrate), and benzamides. No single HDACi is effective against all HDACs. HDACi cause increased acetylation of histone and non-histone proteins and can reactivate p21, which contributes to cell-cycle arrest. Non-cancerous cells are more resistant to the effects of HDACi [75,76].

Epigenetic therapies approved by FDA for leukemia and DNA demethylating agents and HDAC inhibitors, and combinations have been tested in clinical trials [77]. In glioblastoma HDAC inhibitors have entered clinical trials (http://clinicaltrials.gov), while demethylating agents have not been considered [78]. Demethylating agents such as 5-Aza-cytidine or 5-Aza-2 ¢ - deoxcytidine lock DNMT enzymes on to the DNA, thereby inhibiting further DNA methylation. Consequently, demethylating agents require cell division for activity, hence targeting rapidly dividing cells. Due to their unspecific mechanism, demethyling agents may lead to reexpression not only of tumor suppressor genes but also of oncogenes. The HDAC inhibitor vorinostat (SAHA) has shown modest benefit as single agent in a phase II trial for recurrent GBM [79]. The other treatment choise was TMZ and the methylated MGMT promoter sensitizes the tumors to alkylating agents and that the alkylating agent TMZ is part of the current standard of care for GBM [78]. The treatment of GBM patients with valproic acid as antiepileptic drug has shown a survival advantage in combined chemoradiotherapy [80]. Valproic acid is considered to have weak HDAC inhibitor properties and is currently tested in a phase 2 trial for newly diagnosed GBM in combination with standard chemoradiotherapy (NCT00302159). Entinostat, panobinostat phenylbutyrate are the other HDAC inhibitors which are in clinical evaluation for recurrent high-grade gliomaor refractory pediatric brain tumors and neuroblastoma [77]. The AGI-5198 inhibitor identified through a high-throughput screen as a selective R132H-IDH1 inhibitor, induced demethylation of histone H3K9me3 and expression of genes associated with gliogenic differentiation. Blockade of a mutant IDH1 impaired the growth of IDH1-mutant, but not IDH1-wild-type, glioma cells [81].

Conclusion

Necrotic or apoptotic cancer cells releasing genomic DNA and the blood plasma in cancer patients contains DNA derived from these cells and aberrantly hypermethylated genes found in plasma could be one such type of biomarker. This is a less invasive method for biomarker detection in cancer patients. This type of analysis may provide clinically useful information about diagnosis, prognosis and follow-up post-therapy. There is a significant amount of tumour DNA in the plasma of high-grade glioma patients, and in 60% of patients the same methylated promoters (p16INK4a, MGMT, p73, and RARbeta) could be detected in both tumour and plasma DNA [82].

Primary and secondary glioblastoma is a distinct disease subtypes and affecting patients of different age and developing through different genetic pathway. Based on these two subgroups of GBM we may use this different methylation pattern of genes to distinguish between subtypes and aberrant DNA methylation of tumour suppressor genes and oncogenes reported in GBM. The summerized studies mentioned emphasize the relevance of epigenetic gene silencing as a mechanism by which critical biologic pathways are altered in GBM. Several genes discussed above have been identified singly or by genome-wide methylation studies to be epigenetically inactivated. The relationship of aberrant hypermethylation of promoters to the biology of GBM and the prognosis of patients with these tumours is complex and remains to be fully elucidated; it is possible that a more fundamental defect in the epigenetic machinery that regulates promoter methylation may be responsible for the epigenetic changes that cause inactivation of several cancer-related genes and the identification of IDH1/2 mutations in GBM suggests that metabolic pathways may be attractive targets for GBM.

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