Chapter 2

Diffuse Intrinsic Pontine Glioma

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Additional information is available at the end of the chapter

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Abstract

Diffuse intrinsic pontine glioma (DIPG) is a leading cause of brain cancer-related death in children. These aggressive high-grade gliomas cannot be effectively treated and are associated with dismal prognosis. Whilst radiation therapy (RT) prolongs survival, it is a palliative therapy, as half of children with DIPG die within 1 year of diagnosis and almost all are dead by 2 years. These statistics have not changed for decades, despite a multitude of clinical trials. No chemotherapeutic regimen has been shown to improve survival, emphasizing the need to find novel and effective treatments. One of the principal reasons for this poor outcome was our limited knowledge of the biology of DIPG's. Due to their location in brainstem, surgical resection is not feasible and up until recently, even performing a limited biopsy was considered too dangerous. In the last decade, DIPG tumor tissue has become available through autopsies and biopsies. This combined with the genome revolution has resulted in a transformation in our understanding of the underlying biology of this disease. Moreover, viable DIPG cells can now be grown in the laboratory which have allowed development of *in-vitro* (neurospheres) and *in-vivo* models (allograft and xenograft). This chapter summarizes recent advances in DIPG and potential novel therapies.

Keywords: diffuse intrinsic pontine glioma (DIPG), advances in biology, novel therapies

1. Introduction

Diffuse intrinsic pontine glioma (DIPG) is a high-grade glioma originating in the pons and occurs predominantly in children. It is one of the most dreaded pediatric cancers as they are essentially incurable. At the same time, it has become one of the most intense areas of research in the past few years. Due to the lack of tumor samples, there was limited information available about the genetic and molecular abnormalities in DIPG. These tumors were considered

to mimic other pediatric and adult high-grade gliomas and therapies were based on these inaccurate assumptions. Radiation therapy (RT) is the only treatment modality available, that has been demonstrated to prolong survival and improve quality of life (ref) but is not curative. Almost all children die with a median survival of 1 year. Indeed, 50% of children with DIPG die within 1 year of diagnosis and almost all are dead by 2 years (ref). Despite a myriad of clinical trials, no effective treatment has been identified so far. But recently, there has been an exponential increase in the pre-clinical research involving DIPG and many, previously unknown, abnormalities contributing to DIPG pathogenesis have been identified. These may provide treatable targets and help improve the outcome of patients with DIPG.

2. Epidemiology

Tumors of the central nervous system (CNS) constitute the largest group of solid tumors and the second most common pediatric cancer [1, 2]. Around 20% of CNS tumors are brainstem gliomas [3] of which 80% arise within the pons as DIPG [4] with approximately 200–300 children in the United States [5] being diagnosed each year. Survival rates for children with cancer have improved dramatically since the 1960s; from an estimated 5-year survival of 28% to approximately 80–85% at present [6]. The outcome for patients diagnosed with brain tumors has also improved with more than 70% expected to survive at least 5 years from the diagnosis [7]. Pediatric CNS tumors are a very heterogeneous group of diseases with over 100 distinct histological types and survival differs markedly amongst the different histological types. Unlike other childhood cancers, survival for DIPG has not changed [5]. The median survival for children with DIPG is less than 1 year from the diagnosis [8] with more than 90% dying within 2 years of diagnosis [4, 9]. Although DIPG constitute only 10–15% of all pediatric brain tumors, they are the leading cause of death in this group [10]. More than three decades of research and different treatment modalities have not yielded any survival improvement.

3. Clinical features

The symptoms and signs of patients with DIPG occur secondary to the involvement of different parts of the brainstem, which include the midbrain, pons and medulla. The brainstem serves as a conduit through which axonal tracts pass to the spinal cord, cerebrum or exit as cranial nerves. Due to the diffuse nature of DIPG, the tumor infiltrates the white matter affecting the adjacent cranial nerves and white matter tracts [11]. As the pons contains important nuclei critical for life-sustaining function, any damage caused by the tumor or its treatment has devastating effects. DIPG predominantly occurs in in the middle childhood. Median age at diagnosis is 6–7 years, with males and females affected equally [11–13]. Interestingly, adults with DIPG tend to have a longer survival which may indicate a less aggressive and biologically different tumor from that in children [14]. Typically, the presentation is with neurological symptoms of less than 3 months duration [15] with the “classic” triad of cranial

nerve deficits (diplopia and facial asymmetry), long tract signs (hyperreflexia, clonus, upward Babinski, increased tone and decreased strength) and cerebellar signs (ataxia, dysmetria and dysarthria), which is seen in about 50% patients [16, 17]. In most cases, abducens nerve palsy is the earliest sign and is a sensitive predictor for DIPG [17]. Obstructive hydrocephalus with signs of raised intracranial pressure are seen in <10% of patients [18]. Other symptoms for example behavioral changes, night terrors, and school difficulties may also occur.

4. Imaging characteristics

4.1. Magnetic resonance imaging (MRI)

DIPG is diagnosed clinically on the basis of history, clinical signs and MRI findings [18]. The classic MRI appearance is of an expansile lesion centred in the pons that frequently extends laterally into the cerebellar peduncles and hemispheres and often extends vertically into the midbrain and medulla (**Figure 1A**). It is poorly marginated, occupying more than 50% of the axial diameter of the pons [11]. Necrosis can be seen but cysts are rare [14]. The tumors are hypointense with indistinct margins on T1-weighted images (**Figure 1B**) and hyperintense on T2-weighted/fluid-attenuated inversion recovery (FLAIR) images (**Figure 1C** and **D**) [4]. Post-gadolinium enhancement as commonly seen in pilocytic astrocytomas is often minimal or absent in DIPG (**Figure 1E**) [19, 20]. With an average molecular weight of 545 kDa, gadolinium largely exceeds the penetration cut-off of the blood brain barrier (BBB) (400–600 Da) with limited contrast enhancement in DIPG suggesting a largely intact BBB [14, 21]. Other MRI features typical of DIPG include ventral involvement of the pons and encasement of the basilar artery (**Figure 1F**) [5].

4.2. New imaging techniques

4.2.1. MR spectroscopy (MRS)

MRS provides a measure of brain chemistry. The most prominent peaks in the brain spectrum on Proton MRS are N-acetyl aspartate (NAA), creatine, and choline. NAA is a neuronal marker which is usually decreased in tumors. Choline is associated with the metabolism of membrane turnover and is generally increased in tumors. In DIPG, MRS shows a modest increase in choline levels and a decrease in NAA levels [14]. Additionally, peaks from lactate and mobile lipids are often elevated [11]. The abnormalities in these normally occurring brain metabolites may provide insight into the biology of DIPG and become invaluable tools in DIPG radiodiagnosis.

4.2.2. Perfusion and diffusion techniques

Some of the newer perfusion and diffusion MRI techniques are being tested in prospective trials and although not a standard for DIPG diagnosis currently, may prove beneficial in the future.

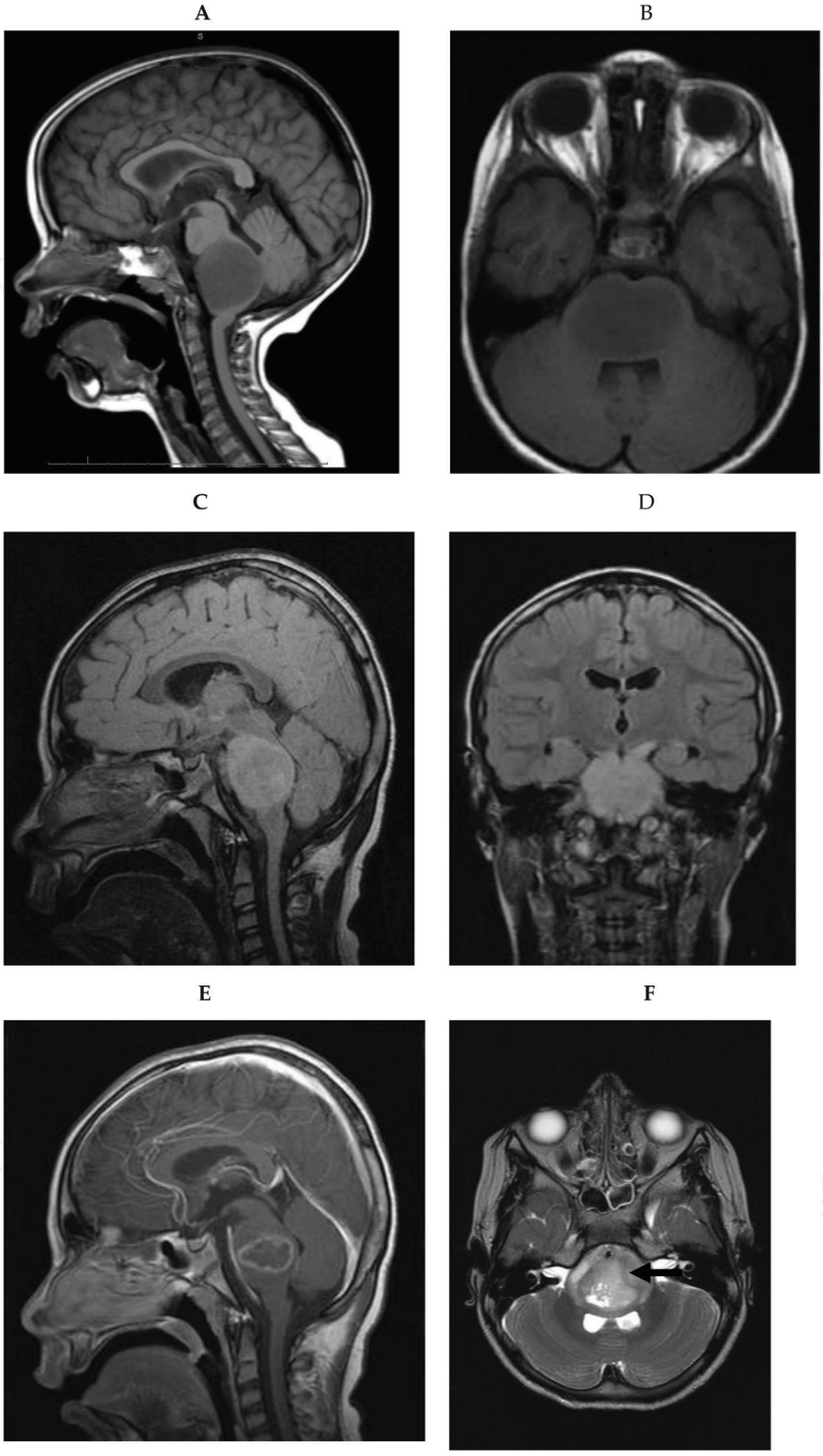

Figure 1. T1-weighted pre-contrast sagittal (A) and axial (B) MRI pictures showing poorly marginated, expansile and hypointense pontine mass. It is hyperintense onT2-weighted/FLAIR sagittal (C) and coronal (D) images. (E) There is minimal post-gadolinium enhancement. (F) Basilar artery encasement by DIPG (black arrow).

MR perfusion measures regional blood volume and flow reflecting the vascular nature of neoplasms [22]. Serial changes in tumor vascularity may be useful to monitor the effectiveness of therapy. Diffusion tensor imaging may provide visualization and quantitative characterization of the major white matter pathways in DIPG [23, 24]. This superior delineation between tumor and normal brain compared to the conventional MRI techniques may prove useful, especially to perform biopsies and obtain DIPG samples. Specific MRI sequences, including single-voxel spectroscopy (SVS), multi-voxel MRS and dynamic susceptibility contrast (DSC) MRI may help in predicting short or long survival interval from diagnosis in patients with DIPG [25].

4.2.3. Molecular drug imaging

Imaging of radiolabeled drugs like monoclonal antibodies and tyrosine kinase inhibitors can be achieved by using PET technology [26]. A recently introduced PET imaging technique of zirconium-89 (89Zr)-labeled bevacizumab in children with DIPG demonstrated considerable inter-and intra-tumoral heterogeneity in drug delivery [27]. Therapeutic potential and toxicity both can be quantified by such non-invasive, in patient techniques of tumor imaging and drug distribution [28]. Thus, these newer imaging modalities provide quantitative physiologic and functional information to complement the anatomic visualization provided by conventional imaging. However, these techniques need further validation and have yet to impact treatment decisions [11].

5. Pathology

Grossly, DIPG tumors tend to spread contiguously, extending to involve the midbrain, medulla, and cerebellar peduncles [29, 30]. Up to 20% of patients are reported to have leptomeningeal disease at diagnosis [31] and almost 56% have spinal metastases or leptomeningeal dissemination at the time of recurrence or autopsy [32, 33]. Microscopically, the majority of tumors resemble malignant gliomas in other regions. Tumor cells appear relatively small, with prominent cytoplasmic intermediate filaments and cell processes [34]. Tumor cells pervade normal cells (**Figure 2A** and **B**), diffusely expanding the pons and distorting, displacing and destroying nerve fiber tracts that normally course through it [34]. Anaplasia, increased mitotic activity (**Figure 2C**), tumor necrosis (**Figure 2D**) and vascular proliferation (**Figure 2E**) are often present [5]. A histopathological hallmark is perineuronal satellitosis in which collection of tumor cells are found around pontine neurons [35]. DIPG is histologically classified as fibrillary astrocytoma, World Health Organization (WHO) Grades II–IV [36] but the prognosis is not associated with histological grade [37, 38]. There can be marked intratumoral heterogeneity with a high proportion of samples showing focal areas of WHO grade I phenotype [39].

5.1. Diffuse midline glioma, H3K27M-mutant

In the latest WHO classification, DIPG have been grouped with other midline gliomas (thalamus, spinal cord) forming a new diagnostic entity. These tumors are characterized by a

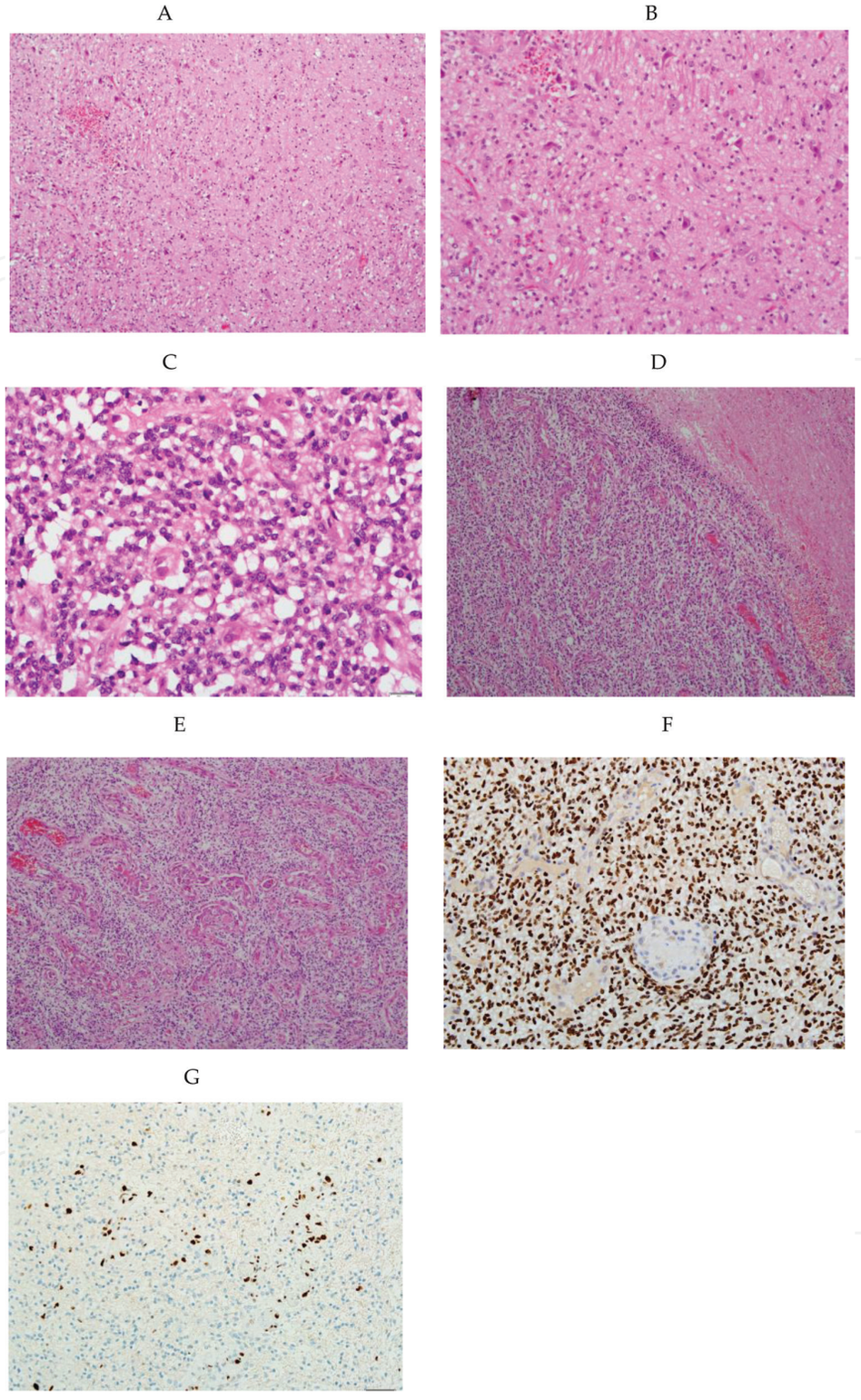

Figure 2. Histologic features of DIPG. Tumor infiltrating medullary neurons, H & E 100× (A) and 200× (B). (C) Tumor showing mitotic figures, H & E 400×. (D) Tumor/necrosis interface showing pseudo-palisading tumor necrosis. (E) Exuberant tumor-associated microvascular proliferation. (F) Tumor cells showing positive nuclear H3K27 M staining with sparing of the endothelial nuclei of admixed blood vessels H3K27 M 200×. (G) Loss of staining of tumor cell nuclei with the trimethylated antibody with retained staining in the endothelial cells, H3K27me3 200×. Images courtesy of Dr. Jason Dyke (Royal Perth Hospital, Australia).

specific histone mutation (H3K27 M) and are called diffuse midline glioma, H3K27M-mutant [40]. A mutation-specific antibody can be used to detect this mutation on immunohistochemistry (**Figure 2F**).

6. The role of biopsy in diagnosis

Prior to the routine MRI use, it was estimated that up to 15% of patients diagnosed with DIPG had a non-glial tumor or non-tumor process [41, 42]. The role of surgical intervention was controversial and, in some centres, biopsy procedures were frequently undertaken for histological confirmation. Although it is not 100% specific, with the wide availability in the early 1990s MRI became the modality of choice for DIPG diagnosis. Biopsy confirmation was thought to be an unnecessary risk as it did not alter the management [43]. The lack of clear biopsy benefit combined with improved diagnostic imaging capabilities led to MRI scans becoming the diagnostic standard of care for DIPG in the United Sates [18, 44]. More recently, there has been a renewed interest in performing stereotactic biopsies in patients with DIPG [45]. In France, biopsies were routinely performed with minimal morbidity and high diagnostic yield [46], nonetheless, it was not a common practice. The increase in the available DIPG tumor samples has yielded valuable data toward improving our understanding of the biology of DIPG [45, 47, 48]. Some of the identified biologic markers have been shown to correlate with progression-free survival (PFS) and may be useful to stratify patients in future clinical trials [49]. Considering the potential for new diagnostic and therapeutic methods combined with low morbidity associated with surgical procedures [50], the option of biopsy is being reconsidered and may eventually be included as part of the routine diagnostic evaluation for DIPG [51]. The second Consensus Conference on Pediatric Neurosurgery recommended biopsy in DIPG to ascertain biological characteristics to enhance understanding and targeting of treatment, especially in the setting of clinical trials [52]. Biopsy from a single area may not be representative of the entire tumor [53] but it may still provide important molecular information.

7. Treatment

More than 250 clinical trials in the last three decades have failed to improve the poor outcome of DIPG [20]. Due to the location it is not amenable to the surgery and chemotherapy agents are yet to show any response. Radiation therapy remains the standard of care at present. The barriers to achieve a cure for DIPG may include the inability of the surgical resection, drug delivery difficulties secondary to the blood-brain barrier (BBB) and intra- and inter-tumoral heterogeneity in the identified molecular aberrations [54].

7.1. Surgery

Surgical resection of DIPG is not feasible due to the presence of vital structures where the tumor is located. Rarely, surgical intervention like ventriculoperitoneal shunt or endoscopic

third ventriculostomy may be necessary to relieve raised intracranial pressure secondary to hydrocephalus. Many patients benefit from steroids which help by reducing peritumoral edema.

7.2. Radiation therapy (RT)

Conventional RT in a dose of 54–60Gy over a 6 week period is commonly utilized in the treatment of DIPG [9]. Temporary improvement or stabilization of symptoms is seen in 70% of patients, but almost all patients have progressive disease. The mean progression-free survival (PFS) is 5.8 months with radiotherapy and 5 months without it [4]. As RT is effective temporarily, non-conventional doses and delivery schedules were investigated. In hyperfractionated RT, the total dose is divided into smaller doses and given more than once a day. Hyperfractionated RT in the form of 1Gy twice a day, to the total dose of 72Gy failed to improve the outcome [37]. Hypofractionated RT is given over a smaller period of time than standard RT by dividing the total RT dose into larger doses and treatments given once a day or less often. Studies investigating the role of hypofractionated RT with 39Gy [55] or 45Gy [56] delivered over 3 weeks have revealed similar survival outcomes, but may be more acceptable to fami9lies due to the shorter delivery times.

7.3. Chemotherapy

Various chemotherapy and targeted agents have been used to potentiate the beneficial effects of RT. These agents were combined before, with, and after RT without much success [9, 10].

7.3.1. Intensive chemotherapy

Chemotherapy combinations used with RT in the setting of phase I–III clinical trials. These include lomustine, vincristine and prednisone [41], cisplatin, etoposide, vincristine, ifosfamide and oral valproic acid [57], myeloablative thiotepa, isotretinoin and vinorelbine [58] and multiple other agents at relapse [59]. One trial evaluated the role of preradiation chemotherapy [60]. The outcome was uniformly poor.

7.3.2. Radiosensitizing agents

i. Temozolomide (TMZ): TMZ is an alkylating prodrug which is converted into its active metabolite monomethyl 5-triazeno imidazole carboxamide. TMZ causes DNA damage by alkylating O^6-guanine, N^7-gaunine and N^3-adenine resides [61]. Due to its proven efficacy in high grade gliomas, low toxicity and radiosensitization potential, temozolomide was trialed to potentiate RT efficacy without much success [8, 62, 63]. Addition of lomustine to adjuvant temozolomide [64] was not beneficial. O6-methylguanine DNA methyltransferase (MGMT) contributes to TMZ resistance by repairing alkylated O^6-guanine nucleotides. But this does not appear to be the cause of TMZ resistance as MGMT is not expressed in DIPG [47]. However, 3-methylpurine-DNA glycosylase (MPG), enzyme

responsible for repair of N^7-gaunine and N^3-adenine nucleotides, and its ATM-dependent regulation may play a role in TMZ resistance in DIPG [65]. A recently closed phase II study stratified patients to receive TMZ and erlotinib based on MGMT methylation and EGFR expression status [66].

ii. Topotecan: topotecan acts as a radiosensitizing agent by stabilizing the DNA topoisomerase I complex, interfering with DNA replication and DNA repair. Concurrent administration of topotecan with RT was found to be ineffective [67, 68].

iii. Other radiosensitizing agents like motexafin-gadolinium [69] and carbogen [70] also showed similar poor results.

8. Biology

More than 250 therapeutic clinical trials including several targeted agents have not improved the dismal prognosis of DIPG [10]. The reason for this, at least in part, has been attributed to our lack of understanding of the biology of this disease. More has been published on the biology and pathophysiology of DIPG in the past 10 years than in all prior years combined [5]. A more recent and significant achievement in DIPG research is sample collection at autopsy. This has provided invaluable insights into understanding of the biology [71, 72]. Both autopsy and biopsy samples have allowed development of *in-vitro* (neurospheres) and *in-vivo* models (allograft and xenograft) [73–75].

8.1. Cell of origin

Pontine precursor-like cells (PPC), found in the ventral pons region, which are positive for the markers for the primitive neural precursor cells, nestin and vimentin, are postulated as the candidate cell of origin for DIPG [35]. Approximately half of PPC also expressed Olig2, a transcription factor which is associated with oligodendroglial precursors. This cell type was morphologically distinct from the nestin positive cells seen in the dorsal brainstem. PPC are present in all ventral brainstem structures during infancy and wane by 2 years of age. The ventral pontine and medullary $nestin^+$ cells show a second peak at 6 years, corresponding to the age of presentation of DIPG. Thus, temporal and spatial distribution of these cells correlates closely with the incidence of DIPG suggesting that tumors arise secondary to dysregulation of a postnatal neurodevelopmental process [35]. Expression of SOX2, a transcription factor with activity during embryogenesis, and Olig2 in another model supports the disordered neurodevelopmental origin of DIPG [76].

8.2. The genomic landscape

DIPG biopsy and autopsy samples have undergone extensive genomic profiling and major breakthroughs have been achieved in identifying key oncogenic pathways [77]. The drivers for DIPG tumorigenesis include epigenetic changes, gene mutations, deletions or overexpression and chromosomal number changes.

8.3. Epigenetic changes

8.3.1. Histone mutations

The DNA is packaged by histone proteins into a chain of nucleosomes which are the basic building blocks of the chromatin fiber [78]. In a single nucleosome, 147 base pairs (bp) of DNA wrap around histone octamers containing two copies each of histones H2A, H2B, H3 and H4 [79]. The N-terminal ends of histones containing lysine (K) and arginine (R) residues are post-translationally modified by acetylation or methylation and regulate DNA repair, replication and transcription. The histone H3 family consists of a number of related proteins. Histone H3 isoforms H3.1 and H3.2 (also called as canonical H3) help in packaging newly replicated DNA. While H3.3 can function much the same as canonical H3 as a core part of the nucleosome, it is also deposited into transcriptionally active regions to replace histones lost during processes disrupting nucleosomes [80].

8.3.1.1. H3 mutations and DIPG

8.3.1.1.1. H3K27M mutations

H3F3A and *H3F3B* produce identical H3.3 proteins whereas *HIST1H3B* is one of the many genes encoding H3.1 [81, 82]. Distinct and recurrent mutations in H3 have been implicated in 70–80% of pediatric gliomas [83]. Lysine to methionine missense mutation at position 27 (K27M) was present in 78% of DIPG patients, with most of these mutations in *H3F3A* and up to 25% in *HIST1H3B* [84, 85]. H3K27M mutations are restricted to the midline structures [86] and H3.1 and H3.3 mutations involve two different oncogenic pathways resulting in distinct clinicopathological variants. H3.1 mutated tumors are exclusively linked to DIPG and exhibit a mesenchymal/astrocytic phenotype, a pro-angiogenic/hypoxic signature and are co-segregated with *ACVR1* mutations. Clinically, these tumors are less aggressive when compared to H3.3 mutant tumors, metastasize less frequently and respond better to radiation therapy (RT) with a median overall survival (OS) of 15 months. H3.3 mutated tumors are located in the midline structures including the brainstem, thalamus and spinal cord. They have a proneural/oligodendroglial phenotype, a pro-metastatic gene expression signature with *PDGFRA* activation. They behave more aggressively, responding poorly to RT with a median OS of 9 months and metastasize more frequently [87]. The gain-of-function H3K27M alterations are exclusive to pediatric high-grade gliomas and any H3 mutation is associated with a dismal outcome but identification of a specific mutation may help in developing specific therapeutic targets.

8.3.1.1.2. G34R/V mutations

H3F3A mutations encoding a glycine 34 to arginine or valine G34R/V comprise a smaller proportion of H3.3 mutations [88]. G34R/V mutations are seen in cerebral hemispheres of slightly older patients (9–42 years) as compared to K27M mutations (5–29 years) [86, 88, 89].

K27M and G34R/V mutations are mutually exclusive and heterozygously expressed, with one wild-type *H3F3A* allele [89].

8.3.1.1.3. Other novel mutations

H3F3A mutation resulting in lysine-to isoleucine substitution at K27 has been rarely seen in DIPG [87]. A mutation in the gene encoding the H3.2 variant, HIST2H3C, resulting in a novel K27 M mutation has been described [87].

8.3.1.2. Downstream effects of H3 mutations and gliomagenesis

8.3.1.2.1. K27 M mutations

K27M (and to a lesser extent K27I) is the only amino acid substitution which can ablate trimethylation (H3K27me3) [90]. The mutant H3K27M binds to the enhancer of zeste homolog 2 (EZH2) component of PRC2 interfering with methyltransferase activity of EZH2 which results in generalized hypomethylation. The downstream effect is derepression of targets of PRC2, upregulation of gene expression and gliomagenesis [91]. In addition, K27M mutation contributes to altered cell cycle control, inhibition of autophagy pathways and potentially increased resistance to radiotherapy [92]. Even though the mutant histone forms only 3.6–17.6% of the total cellular H3 pool, there is a near-absolute loss of H3K27me3. This represents a *trans*-dominant-negative effect across all three isoforms of the wild-type H3 protein [90, 91, 93, 94]. However, the exact role of H3K27M in DIPG tumorigenesis remains unknown as it does not induce the tumors on its own *in vivo* [95]. One of the postulates of gliomagenesis is H3.3 K27M and G34R/V acting as driver mutations followed by a second hit by another mutation like *TP53, PPM1D, ACVR1 or PI3KR1* [83, 95, 96].

In summary, H3K27 mutations have a great significance irrespective of whichever histone H3 variant (H3.1-HIST13B, H2-HIST2H3C and H3.3-H3F3A) is targeted and will result in loss of H3K27me3 and development of DIPG.

8.3.1.2.2. G34R/V mutations

The role of H3.3G34R/V in gliomagenesis is less clear. It may act by disrupting K36me3 levels and activating potential oncogenes [90, 93]; inducing *MYCN* upregulation [97, 98] and disrupting interaction between H3.3 and ATRX/DAXX leading to aberrant deposition of H3.3 near telomeric regions and leading to alternate lengthening of telomerase [88, 89, 99].

8.3.1.3. Co-mutations associated with H3 mutations in DIPG

Other mutations associated with H3K27M mutation include α thalassemia/mental retardation syndrome X-linked (*ATRX)* or death-domain associated protein *(DAXX)* (30%), *TP53* (60%) and *NF-1*, *PDGFRA*, *BRAF*, *KRAS*, and *FGFR1* at lower frequencies [88, 99, 100]. G35R/V mutations coexpress with mutations in *TP53*, *ATRX*/*DAXX* and *PDGFRA* [83].

8.3.1.4. Mutations of chromatin modifiers

Chromatin writers or erasers are the enzymes which catalyze the post-translational modifications of histone tails like methylation, acetylation and ubiquitylation of lysine residues,

phosphorylation of serine or threonine residues and methylation of arginine residues. The effector proteins called readers are recruited to the chromatin by the resultant histone code which helps in localization of functional complexes that affect transcriptional regulation [101]. Numerous recurrent mutations are observed in chromatin writers, erasers, readers and remodelers in DIPG and other tumors [102].

The discovery of histone mutations which are present in up to 80% of DIPG is one of the most remarkable breakthroughs in terms of understanding DIPG biology and identification of actionable targets [90, 92, 99].

8.3.2. Polycomb repressive complex (PRC) abnormalities

Polycomb group proteins remodel chromatin enabling epigenetic silencing of genes. There are two main Polycomb group complexes found in mammals-PRC1 and PRC2. PRC1 catalyzes the monoubiquitylation of histone H2A and PRC2 catalyzes the methylation of H3K27 [103]. Some PRC1 complexes also act independent of enzymatic activity to regulate gene expression by compacting chromatin [104]. PRC1 functions downstream of PRC2 by binding specifically to H3K27me3 [103]. By inducing such sequential histone modifications, PRC1 and PRC2 achieve stable silencing of gene expression [105]. Dysregulation of PRC and its downstream targets has been implicated in many cancers [83]. B cell-specific Moloney murine leukemia virus integration site 1 (BMI-1) is a component of PRC1 complex. It was found to be highly expressed in DIPG tumor cells and its downregulation inhibited various cellular processes like cell proliferation, cell cycle signaling, telomerase expression and activity, and cell migration [105].

8.4. Gene abnormalities

Molecular profiling of DIPG samples has provided new insights [61]. In the past, candidate gene approaches were utilized to identify gene abnormalities associated with adult high-grade gliomas (HGG) [106]. Although, these studies were limited in defining the biology of DIPG due to their small numbers, they still highlighted some differences between adult HGG and DIPG. Recently, studies performed with next-generation sequencing approaches have confirmed that DIPG are molecularly distinct from adult HGG and non-DIPG pediatric HGG [18]. The current technologies utilize whole-genome sequencing (WGS), whole-exome sequencing (WES), and RNA-sequencing in addition to copy number, gene expression, and methylation profiles and histopathology.

8.4.1. Mutational burden of DIPG compared to other tumors

The genomic signatures of the most pediatric HGG are complex and involve significant copy number alterations (CNAs), single nucleotide variants (SNVs) and structural variants [107–109]. HGG have a higher mutation burden than many other pediatric cancers but it is still lower than common adult cancers [77]. HGG commonly show structural variants like simple rearrangements and abnormalities caused by chromothripsis [84]. But there is a wide range of genomic complexity in pediatric HGG. At one end of the spectrum is infant non-brainstem HGG (NBS-HGG) arising in children less than 3 years old. These tumors have significantly

lower mutation burden [84]. At the other end of the spectrum are HGG from patients with inherited mutations in mismatch repair genes. Germline mutations in tumor suppressor genes like *TP53* and neurofibromin 1 (*NF1*) predispose to the development of HGG [110]. The hypermutated tumors arising in the context of these germline mutations show a very high number of somatic SNVs; these may be more than 100-fold higher than in 95% of pediatric HGG [84]. DIPG show similar mutation burden as other pediatric HGG and their genomic complexity is indicative of multiple genetic mechanisms generating numerous mutations which provide the tumor with diverse potential pathways to therapeutic resistance [77].

8.4.2. Abnormalities of cellular proliferation pathways

8.4.2.1. Bone morphogenetic protein (BMP) signaling and ACVR1 mutations

ACVR1, also known as *ALK2* encodes the serine kinase, Activin receptor type 1 A (ACVR1) [111]. It is a type 1 BMP receptor which belongs to the mammalian TGF-β signaling family [112]. ACVR1 binds to a diverse set of ligands, including TGF-β, activins and multiple BMP [113]. ACVR1 is essential for signaling and after ligand it is phosphorylated by ACVR2 with formation a stable ACVR1/2 complex [114]. This results in the phosphorylation and activation of growth promoting genes through SMAD transcription factors [77]. ACVR1 mutations are constitutionally activating, leading to increased expression of activin signaling targets *ID1* and *ID2* [115].

8.4.2.1.1. ACVR1 mutations and DIPG

Germline *ACVR1* mutations cause the congenital malformation syndrome fibrodysplasia ossificans progressive (FOP) [116]. Seven somatic mutations of this gene have been identified in 13–32% of DIPG samples leading to either ligand-independent kinase activation or gain-of-function effects [84, 111, 112, 115]. However, there is no increased cancer risk in FOP despite having similar germline mutations as those seen in DIPG which suggests that *ACVR1* mutations on their own are not tumor initiating and lead to DIPG only in the presence of other mutations [77, 116].

8.4.2.1.2. ACVR1 co-mutations

ACVR1 mutant tumors commonly co-segregate with *HIST1H3B* mutations [115]. *ACVR1-HIST1H3B* co-segregating tumors do not show *TP53* loss or *PDGFRA* amplifications but around 60% have mutations in the PI3K signaling pathway [114].

8.4.2.1.3. Clinical implications

ACVR1 mutations signify a distinct subset of DIPG patients. They occurred more frequently in females (F:M ratio of 1.75:1) and are associated with younger age and longer survival (median OS of 14.9 months) [84, 111]. There was a significant pharmacologic inhibition of ACVR1 by a selective ALK2 inhibitor, LDN-193189, leading to dose-dependent cytotoxicity across all the tested DIPG cell lines [111]. Due to its role in DIPG pathogenesis and targetable potential, ACVR1 inhibition represents a novel therapeutic option.

8.4.2.2. Receptor tyrosine kinase (RTK) pathway

RTKs are transmembrane protein receptors containing intrinsic enzymatic activity. Their ligands include growth factors, hormones and cytokines [117] and they play an critical role in mediating key signaling pathways involving cell proliferation, differentiation, survival and migration [118]. The human RTK family has 20 subfamilies and 58 known members including platelet-derived growth factor receptors (PDGFR), epidermal growth factor receptors (EGFR) and fibroblast growth factor receptors (FGFR) [118–120]. Upon ligand binding, the RTKs are activated leading to signal transduction to the nucleus and subsequent protein transcription. This is achieved by downstream activation of various RTK substrates like mitogen-activated protein kinase (MAPK) and phosphatidylinositol-3-kinase (PI3K) [121].

8.4.2.2.1. RTK pathway aberrations and DIPG

Amplifications and mutations in components of RTK-RAS-PI3K pathway are seen in up to 60% of DIPG [18]. The most common affected component is *PDGFRA*. Other abnormalities involve *AKT1*, *AKT3*, *c-MET*, epidermal growth factor receptor (*EGFR*), erythroblastic leukemia viral oncogene homolog-4 (*ERBB4*), hepatocyte growth factor, Kirsten rat sarcoma viral oncogene homolog (*KRAS*), *PIK3CA*, *PIKC2G*, *PIK3R1*, *PTEN*, insulin-like growth factor 2, and insulin-like growth factor receptor [18, 45, 53, 111].

8.4.2.2.1.1. PDGFRA amplifications

Whole-genome profiling of DIPG tumors have identified recurrent amplifications of *PDGFA* and *PDGFRA* with overexpression of PDGFR-α in 28–50% tumors [47, 48, 122, 123].

8.4.2.2.1.2. PDGFRA mutations

Somatic activating mutations including missense mutations and in-frame deletions and insertions were identified in 4.7% DIPG tumors and were found to be oncogenic *in vivo* [124]. Concurrent amplification was seen in 40% of tumors with mutations and 60% had heterozygous mutations [124]. Similar mutations were identified in other studies in 8.8–25% samples [54, 123]. Downstream activation of the PDGFR pathway has been shown by phospho-mammalian target of rapamycin (m-TOR) immunopositivity [47] as well as activation of MAPK and PI3K pathways [124]. PDGFR-α is expressed by the oligodendrocyte precursor cell derived from the candidate cell of origin PCC [35] and hence the precursor cell may be responsive to PDGF. Also, human DIPG cell culture yield was better after the addition of PDGF [35] and upregulation of PDGF pathway was associated with dorsal pontine glioblastoma in mouse models [125, 126].

8.4.2.2.1.3. EGFR aberrations

EGFR immunopositivity and gene amplification were seen in about 27% [47] and 7–9% [48] of cases respectively.

8.4.2.2.2. RTK pathway co-mutations in DIPG

PDGFRA gains and amplifications co-segregate with H3.3 mutations [88, 99] and *TP53* mutations [124].

8.4.2.2.3. Clinical significance

RTK signaling dysregulation, particularly PDGFR pathway either overexpression or mutation, may have an important role in the pathophysiology of DIPG and provide therapeutic targets [47]. Identification of PDGFRA mutations may be beneficial in developing targeted therapies. But some particular mutations like PDGFR D842V in the gastrointestinal stromal tumors confers resistance to imatinib [127]. Others may have only cytostatic effects so they may not be effective on their own [124].

8.4.2.3. MYC and MYCN aberrations

MYCN proto-oncogene is a member of the MYC family encoding the protein MYCN. MYCN plays a critical role during embryogenesis and is involved in cellular proliferation and differentiation [128]. *MYCN* amplification is seen in DIPG [84, 92, 99] and is associated with hypermethylation, high-grade histology and chromothripsis on chromosome 2p [115]. *MYCN* amplifications are transcriptional regulators that affect the epigenetic landscape by enhancing gene expression across the whole genome [84]. MYCN pathway maybe induced by H3.3 K27 M [99] or H3.3G34V [83, 102] but may act independent of H3 mutations [92].

8.4.2.4. Hedgehog (Hh) signaling

Hh signaling pathway plays a major role in regulation of developmental processes like cell proliferation, cell differentiation, tissue polarity and stem cell maintenance. Aberrant activation of Hh pathway has been implicated in the pathogenesis of cancers like medulloblastoma. No structural mutation involving this pathway resulting in the development of DIPG has been identified so far. However, in pre-clinical murine models [35] upregulation of Hh pathway activity resulted in ventral pontine hyperplasia. Hh pathway is essential for the normal development of PPC in humans. Also, Hh pathway activation stimulates and blockade reduces the self-renewal capacity of DIPG neurosphere cells. These findings indicate that Hh signaling, which drives the development of neural precursors in the ventral pons, may play a role in tumor formation in a subset of DIPG. Patients with Gorlin syndrome, a genetic entity occurring secondary to unregulated Hh activity, usually do not develop DIPG [35]. So, in addition to unregulated Hh pathway activity, a second "hit" may be necessary for DIPG transformation. Hh signaling role in the pathogenesis of DIPG was further investigated in a study [92] which identified upregulation of Hh signaling. DIPG samples showed upregulation of Patched (PTCH) and nuclear translocation of Glioma Associated Oncogene 1 (GLI1); both PTCH and GLI1 are key Hh pathway molecules. In summary, Hh pathway may play a significant role in DIPG tumorigenesis by stimulating PPC and transforming them into potential DIPG cancer stem cells (CSC).

8.4.3. Abnormalities of cell cycle regulation pathways

8.4.3.1. TP53 pathway

The TP53 pathway is a complex network of genes which respond to diverse internal and external stress signals and have an impact on the normal cellular homeostasis [129]. The p53 protein is activated by stress signals transmitted as post-translational modifications leading to apoptosis [130]. In addition, the TP53 pathway produces proteins which aid directly in DNA repair processes and alter cellular environment enabling inter-cellular communication [131]. In the critical role of safeguarding the genomic integrity, it functions as a tumor suppression pathway [132]. *TP53* is the most commonly mutated gene found in a broad variety of human cancers [129, 133].

8.4.3.1.1. TP53 mutations and DIPG

TP53 mutations are commonly found in DIPG with the reported incidence between 9 and 77% [45, 99]. They are more common in higher grade histology tumors (grades III and IV) [53]. About 50% of *TP53* wild-type grade II DIPG show presence of *PPM1D* mutations [134]. *PPM1D* is an oncogene associated with cancers like neuroblastoma [135] and lung cancer [136] which codes for wild-type p53-induced phosphatase 1D (WIP1). WIP1 is a negative regulator of *TP53* as it inactivates p53 and promotes termination of stress-induced responses. So *PPM1D* mutations have the same functional significance as *TP53* mutations [137]. *PPM1D* and *TP53* mutations are mutually exclusive and may ultimately lead to dysregulated homeostasis and tumorigenesis [134].

8.4.3.1.2. TP53 co-mutations in DIPG

TP53 mutations more commonly co-segregate with H3.3 K27 M than H3.1 K27 M [112] and frequently occur in the setting of *PDGFRA* aberrations [124].

8.4.3.2. The RB pathway

Cyclins and cyclin-dependent kinases (CDKs) control the G_1/S transition of the cell cycle [138]. The abnormalities involving these regulators observed in DIPG include cyclin-dependent kinase inhibitor 2A or 2B (*CDKN2A* or *CDKN2B*) deletions [48, 122] and *CDK4*, *CDK6* or cyclin D1 (*CCND1*), *CCND2,* and *CCND3* amplifications [48, 122, 139].

8.4.3.3. Aurora kinase pathway

Aurora kinase family include three highly homologous serine/threonine kinases required during mitosis and which are linked to many cancers [140]. AURKB forms the catalytic component of the chromosomal passenger complex (CPC) which plays a critical role during mitosis [141]. Almost 70% of DIPG have demonstrated overexpression of *AURKB* [142].

8.4.3.4. WEE1 kinase pathway

WEE1 kinase is an important part of G_2 checkpoint. DIPG cells, unlike normal cells, have aberrations in genes regulating the G_1 checkpoint, including *TP53*, *MDM2*, *CDKN2A*, and *ATM*

[122, 133, 143] causing a dysfunctional G_1 arrest. So, these cells rely heavily on G2 checkpoint to repair DNA damage caused by irradiation. WEE1 protein is significantly overexpressed in post-mortem DIPG samples [73]. Abrogation of the G_2 checkpoint achieved by WEE1 kinase inhibition pushes DIPG cells with unrepaired DAN damage into mitotic catastrophe resulting in cell death.

8.4.3.5. Poly (ADP-ribose) polymerase (PARP)-1 abnormalities

PARP-1 is a nuclear protein involved in the DNA damage repair processes [144]. PARP-1 activity provides an escape mechanism for cancer cells to avoid apoptosis and its overexpression may be associated with temozolomide and radiation resistance [47]. Gain of *PARP-1* is seen in DIPG tumors and provides a potentially targetable therapeutic option [47].

8.5. Chromosomal number abnormalities

Copy number abnormalities (CNAs) reported in DIPG include gain in chromosomes 1q, 2q, 8q, 9q, 7p/7q and loss in chromosomes 16q, 17p, 20p, 21q, 10q and 4q [47, 48, 122, 123, 139, 145]. The CNAs may represent the initial mutations responsible for DIPG tumorigenesis as well as the treatment effect.

8.6. Immune checkpoint abnormalities

8.6.1. B7-H3 abnormalities

B7-H3 or CD276, a member of the B7-CD28 family, is a type I transmembrane glycoprotein [146]. Many malignant neuroectodermal tumors including adult HGG over-express B7-H3. B7-H3 was found to be overexpressed in a small panel of DIPG samples obtained at autopsy [147]. A monoclonal antibody 8H9 recognizes it and binds specifically to the tumor cells [148, 149] enabling therapeutic cell selectivity. B7H3 was targeted safely in the salvage therapy of stage IV neuroblastoma using intrathecal ^{131}I-8H9 [150].

8.7. Tumor microenvironment abnormalities

Therapies targeted at intrinsic cellular pathways have yielded poor results in DIPG. Tumor microenvironment plays a vital role in tumorigenesis and progression, so studies have looked into investigating microenvironment alteration for better results.

8.7.1. Neuroligin-3 (NLGN3) role

Neuroligin-3 (NLGN3) is a synaptic adhesion molecule which is cleaved from neurons and oligodendrocyte precursor cells via the ADAM10 sheddase and released into the tumor microenvironment. This important neuronal activity promotes many types of brain cancers including DIPG, pediatric and adult HGG and anaplastic oligodendroglioma. NLGN3 release activates oncogenic pathways like focal adhesion kinase activation resulting in the downstream PI3K-mTOR pathway induction. This in turn causes upregulation of several synapse-related genes resulting in the proliferation of glioma cells [151]. HGG glioma growth in xenograft models was blocked by ADAM10 inhibitors by preventing NLGN3 release into

the tumor microenvironment. Similarly, patient-derived orthotopic xenografts fail to grow in *Nlgn3* knockout mice [152].

8.7.2. Pleiotrophin (PTN) role

PTN is a growth factor secreted by neural precursor cells in the lateral ventricle subventricular zone (SVZ). It has an important role in normal neurodevelopment, plasticity and regeneration. PTN acts as a glioma chemoattractant. Through autocrine/paracrine actions, it activates Rho/Rho kinase (ROCK) signaling pathway enabling migration of DIPG cells to the SVZ [153].

9. DIPG subgroups

Proteomic, methylation and mRNA analyses have identified interesting subgroups of DIPG.

PDGFRA amplification is seen in association with H3.3 mutation and ACVR1 mutation is mainly seen in H3.1 mutant tumors [87, 115].

Upregulation of N-Myc and Hh signaling pathways [92].

Upregulation of N-Myc (H3 wild-type), silent genome and H3K27 M [115].

Mesenchymal transition with stem cell-like phenotype and oligodendroglial differentiation and PDGFRA amplification/mutation.

10. Novel therapeutic approaches

10.1. Molecularly targeted agents

10.1.1. RTK-RAS-PI3K pathway

Given DIPG tumors show aberrant activation of growth factor receptor-mediated signal transduction pathways, using drugs targeting these pathways is a rational approach. *In-vitro* studies confirmed the efficacy of tyrosine kinase inhibitors (TKI) like dasatinib in reducing tumor proliferation and inhibition of *PDGFRA* activity [154]. Phase I studies of PDGFR pathway inhibition by imatinib [155] and dasatinib [156], VEGFR2 inhibition by vandetinib [156, 157], EGFR inhibition by gefitinib [158] and erlotinib [49] revealed the safety of using these drugs in children and provided the doses for phase II studies. Temsirolimus is an mTOR inhibitor [159] and its combination with perifosine, an Akt inhibitor, was shown to be safe and feasible in a phase I study in children with recurrent/refractory solid tumors including DIPG [160]. This study tested the hypothesis of dual targeting of PI3K-Akt–mTOR pathway. Phase II studies of multi-tyrosine kinase inhibitor sunitinib [161] and farnesyl transferase inhibitor tipifarnib, which inhibits farnesylation of Ras, [162] did not show activity. BIOMEDE, a phase II study, is stratifying patients based on overexpression of EGFR and/or loss of PTEN following diagnostic biopsy. The patients are randomized or assigned to different treatment arms with erlotinib, everolimus and dasatinib [163].

10.2. Epigenetic modifying agents

Histone deacetylases (HDAC) regulate the histone acetylation in nucleosomes, which mediates changes in chromatin conformation, leading to gene expression regulation [164]. HDAC gene mutations, downregulation and altered expression are linked to tumorigenesis. Histone deacetylase (HDAC) inhibitors modify histone activity to increase expression of previously silenced genes thereby leading to cell death [165]. Panobinostat is a multi-HDAC inhibitor which increases global H3 acetylation and H3K27 M methylation and reduces oncogene expression. Panobinostat-induced polyacetylation of the H3 N-terminal tail has been shown to reverse PRC2 inhibition caused by K27 M and rescue the H3K27 hypomethylation phenotype [166]. Panobinostat has shown promising results in *in vitro* [164] and some *in vivo* [167] studies although a narrow therapeutic index causing dose limiting toxicities at the required antitumor concentrations being the likely reason for lack of efficacy [164]. Its efficacy was further enhanced by histone demethylase inhibitor GSK-J4 which increase H3K27me3 [168]. DIPG patients showed no objective responses to single agent vorinostat in a phase I clinical trial [169]. Vorinostat is currently being investigated in combination with temsirolimus and RT in DIPG in a phase I trial [170]. Pediatric Brain Tumor Consortium (PBTC) is undertaking a phase I trial of single agent Panobinostat in patients with DIPG [171].

10.3. Targeting cell cycle regulation

DIPG tumors contain cell cycle regulatory gene abnormalities like amplification of D-type cyclins and CDK4/6 or loss of Ink4a-ARF resulting in cellular proliferation [172]. Targeting the cyclin/CDK/RB pathway was investigated in a preclinical trial. PD-0332991 (PD), a CDK4/6 inhibitor, was used in a genetically engineered, PDGF-B overexpressed, Ink4a-ARF and p53 deficient brainstem glioma mouse model. PD induced cell cycle arrest both *in vitro* and *in vivo*. The survival of mice treated with PD alone or in combination with RT was significantly more than untreated or mice treated with only RT [172]. Inhibition WEE1 kinase, which is overexpressed in DIPG, was shown to increase the RT response in vitro and in vivo [73].

10.4. Immunotherapy

Interferon, IL-12 and anti-glioma monoclonal antibody have shown efficacy in mouse models of non-brainstem malignancies [173]. Recently, subcutaneous vaccinations with glioma-associated antigen epitope peptides (EphA2, IL13-Rα2 and survivin) were investigated in children with DIPG. The vaccine was tolerated well and the results were encouraging for future trials [174]. IL13-Rα2 is of particular significance as it is highly and differentially expressed in DIPG [175, 176] compared to the normal brain tissue which makes it a suitable immunotherapy target.

10.5. Human neural and mesenchymal stem cells

Neural stem cells (NSC) have been utilized as the vehicle for therapeutic agents in gene therapy for experimental malignant brain tumors [177, 178]. NSC have robust tumor tropism and this property can be used to deliver therapeutic agents to the inoperable DIPG tumors. NSC are difficult to harvest and use due to their inherent immunogenicity [179]. Mesenchymal

stem cells (MSC) have been found to be a suitable alternative as they have all the properties of NSC and are more practical to use [180]. In a rat model, brainstem glioma directed migratory capacities of NSC and MSC from different sources was found to be similar *in vitro* and *in vivo*. This study showed promise as 20–30% of stem cells migrated from the site of injection in the right forebrain to the brainstem glioma cells. In addition, the group treated with genetically engineered NSC encoding cytosine deaminase (CD) suicide gene and IFNβ proinflammatory cytokine and systemic 5-fluorouracil resulted in 59% reduction in the tumor size [181].

10.6. Delivery methods to overcome blood-brain barrier (BBB)

The vast majority of brain tumors are characterized by the loss of BBB integrity due to the disordered and highly permeable tumor neovasculature [182]. In DIPG, however, the BBB integrity is normal and the drug permeability is reduced as the tumor makes use of the existing brain vasculature [183]. Traditional cytotoxic drugs have shown good efficacy against DIPG cells *in vitro* but they likely fail to penetrate the BBB which may explain the contrasting results with clinical trials [28]. To overcome the disappointing results associated with systemic therapy, methods utilizing localized and targeted drug delivery have been investigated.

10.6.1. Convection-enhanced delivery (CED)

CED is a relatively novel delivery modality which utilizes the properties of bulk flow to achieve homogeneously distributed infusions into the brain parenchyma [184]. It is a neurosurgical technique in which one to several catheters are stereotactically placed within or around the tumor mass and drug is delivered locally, bypassing the BBB and reducing systemic toxicity [185, 186]. The local concentrations achieved are significantly higher than the systemic administration and the drug distribution occurs along the patterns of the glioma cell invasion along the white matter tracts [187]. Many *in vivo* feasibility studies have confirmed that drugs can be safely delivered into the rodent brainstem [188]. CED of carmustine [74, 189], carboplatin [190, 191], temozolomide [192], small-molecule kinase inhibitors [193], cisplatin [194] and gemcitabine [190, 195] have been evaluated for safety and distribution parameters. Topotecan CED in two pediatric patients with DIPG showed initial reduction in tumor size [196]. MRI guided and robotically placed catheters were used for CED of carboplatin in DIPG patients. Three out of eight patients survived beyond 15 months and the procedure was tolerated well [197, 198]. Administration of ^{124}I-8H9, a radioactively labeled antibody and a chimeric toxin with B7-H3 specificity, is currently being explored in a phase I clinical trial for DIPG patients [199]. A patient with DIPG received CED infusion of the recombinant cytotoxin IL13-PE38QQR with GD-diethylenetriamine but the tumor progresses within a few weeks [200]. IL13-PE38QQR is a recombinant *Pseudomonas aeruginosa* toxin and some early phase clinical trials investigating its CED efficacy are being conducted or recently completed in patients with DIPG and HGG [185]. These studies have established the feasibility of CED. However, more data is required to prove safety and efficacy and CED models are undergoing rigorous investigations into the physical properties of the catheters and infusion rates [173]. CED targets the primary tumor only and not the metastatic sites. In DIPG, local and distant metastases in the brain or spine are seen in 13–17% patients. Therefore, in situations where disseminated disease is present, CED therapy alone, directed toward the primary tumor site, will be inadequate and should be complemented with therapies for the secondaries [28, 32].

10.6.2. Nanoparticle delivery

Another alternative drug delivery technique involves encapsulation of cationic substances into capsules termed micro- and nanoparticles. This reduces their tissue affinity and increases the volume of distribution [28, 201].

10.6.3. Cell-mediated delivery

Bacterial cell-derived vehicles to transport chemotherapy agents across the BBB was utilized with bi-specific antibodies recognizing cell wall and EGFR moieties [202]. Similar EGFR-targeted vehicles loaded with doxorubicin resulted in significant tumor regression in canine brain cancer models [203]. ECREST (A Study of Intravenous EEDVsMit in Children With Recurrent/Refractory Solid or CNS Tumors Expressing EGFR) is a phase I study using mitoxantrone loaded nanocells in EGFR positive relapsed/refractory solid and CNS tumors including DIPG [204].

10.6.4. BBB disruption techniques

BBB can be temporarily disrupted by ultrasound methods. This results in enhanced uptake of systemically delivered drugs [28].

11. Conclusion

DIPG remains the main cause of death in pediatric patients with brain tumors. Despite many clinical trials, minimal improvement has been achieved as compared with RT. Most of the clinical trials in the past did not have sound biological bases due to a lack of biopsy specimens. In the last few years, international collaborative research has helped to identify new molecular aberrations in DIPG. These have provided insight into the pathogenesis of DIPG and may help in identifying new targeted therapies.

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