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Review

Engineered extracellular vesicles (EVs): Promising diagnostic/therapeutic tools for pediatric high-grade glioma

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ABSTRACT

Diffuse intrinsic pontine glioma (DIPG) is a highly malignant brain tumor that mainly occurs in children with extremely low overall survival. Traditional therapeutic strategies, such as surgical resection and chemotherapy, are not feasible mostly due to the special location and highly diffused features. Radiotherapy turns out to be the standard treatment method but with limited benefits of overall survival. A broad search for novel and targeted therapies is in the progress of both preclinical investigations and clinical trials. Extracellular vesicles (EVs) emerged as a promising diagnostic and therapeutic candidate due to their distinct biocompatibility, excellent cargo-loading-delivery capacity, high biological barrier penetration efficiency, and ease of modification. The utilization of EVs in various diseases as biomarker diagnoses or therapeutic agents is revolutionizing modern medical research and practice. In this review, we will briefly talk about the research development of DIPG, and present a detailed description of EVs in medical applications, with a discussion on the application of engineered peptides on EVs. The possibility of applying EVs as a diagnostic tool and drug delivery system in DIPG is also discussed.

1. Introduction

As one of the most common malignant tumors occurring in children, diffused intrinsic pontine glioma (DIPG) is the main reason for the death of pediatric brain tumor patients [1]. The median survival of DIPG is less than one year, and the dismal 5-year overall survival (OS), is less than 1 % and independent of the treatment received [2–4]. Unlike high-grade glioma (HGG) which affects mostly adults, the median age at the onset of DIPG is 6–7 years [5–7]. Patients with DIPG generally present clinical symptoms including cranial nerve palsy, pyramidal tract dysfunction, cerebellar signs, or long-tract signs [8,9]. Other non-specific symptoms include behavioral abnormalities, sensory problems, and urinary issues [8].

Over the years, the diagnosis of DIPG was based on clinical signs and neuroimaging only (such as computed tomography (CT) or magnetic resonance imaging (MRI)) because the tumors are nonresectable (Fig. 1). Although not routinely applied, tissue biopsy is recommended for DIPG diagnosis since tissue biopsies provide abundant molecular information, thanks to the highly developed sequencing technologies [10–12]. Molecular parameters-based diagnosis, has, therefore, shed light on the precise classification of central nervous system (CNS) tumors since 2016 [13,14]. DIPG, as a unique subtype of glioma, is belonging to diffuse midline glioma (DMG) with a mutation of lysine to methionine at site 27 of histone 3 (H3K27M). The updated 2021 fifth world health organization (WHO) CNS classification updated "H3K27M" with "H3 K27-altered", implying that multiple molecular mechanisms are

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involved [14]. Currently, it is still difficult to perform surgery due to the special location and highly diffused features of the tumor. Although conventional radiotherapy is considered a palliative treatment [2], the high recurrence and serious side effects caused by radiotherapy cannot be ignored. Up to now, there's no approved drug worldwide for the precise treatment of DIPG. Therefore, there is an urgent need to develop novel therapeutic strategies addressing this incurable disease.

There are a couple of novel strategies to combat DIPG in recent years. While immunotherapy and inhibitors therapy remain to improve the outcomes of DIPG, nanotherapy can be considered as another option, as nanotherapy started to show benefits for many brain diseases [17–20]. Extracellular vesicles (EVs), one of the best representative nanotherapy, are a group of membranal-containing nanovesicles produced by all kinds of cellular organisms. The term EVs is used to describe subgroups that include among others: exosomes, which are released in the extracellular space by multi-vesicular bodies upon fusion with plasma membrane, microvesicles or ectosomes that are directly shed by the plasma membrane [21,22]. Noteworthy that the subsets of EVs – microvesicles, exosomes, and apoptotic bodies have overlapping features (size, charge, density, surface marker), making it difficult to distinguish them [23–26]. EVs from different sources inherit a variety of properties, such as yield, content, and function. This highly heterogeneous character ensures EVs hold the unique ability to induce complex biological responses [27–31]. Beyond the important role in biological processes, EVs have displayed significant contributions to the progress of brain diseases since they are capable of crossing the blood-brain barrier (BBB) or blood-brain-tumor barrier (BBTB) [32,33]. Therefore, it is reasonable to consider EV as a promising therapeutic tool to combat DIPG.

The role of EVs as the advanced delivery vehicle for DIPG has not been widely reported due to limited resources. The aim of this review was, therefore, probably the first to highlight the potential of utilizing EVs for DIPG diagnosis and therapy. We will briefly summarize the current understanding of DIPG and provide an overview of how novel technologies are being applied in DIPG first. While the biological functions of EVs have been extensively described in different reviews [27, 34–41], this review, on the other hand, will then focus on the medical application of EVs in cancers and brain diseases, and discuss one of the future directions of the EVs field – for improving DIPG care.

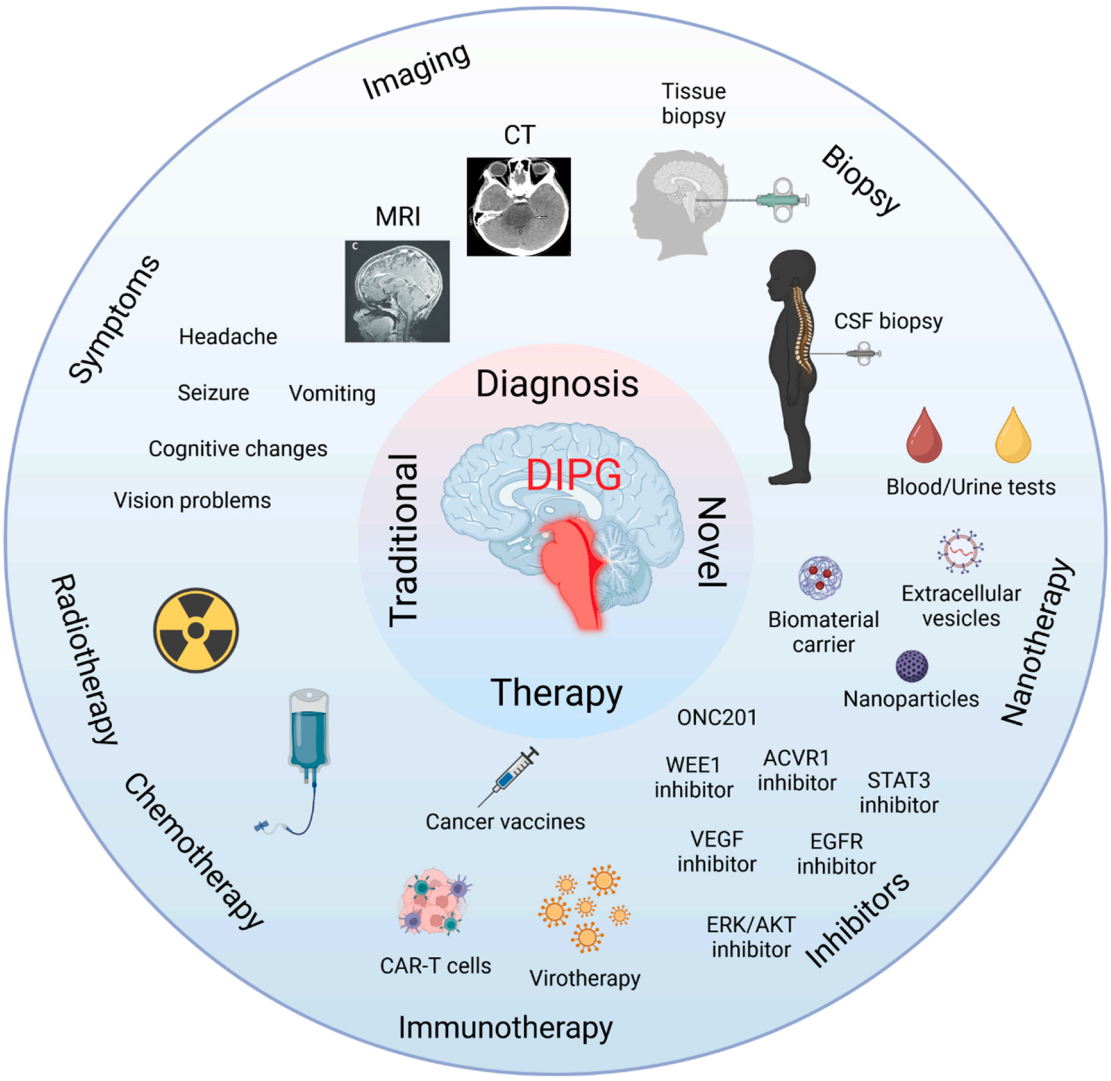

Fig. 1. The diagnosis and therapy of DIPG through traditional and novel strategies. The clinical images are recreated based on Himes et al. [15] and Radiology Key [16]. Abbreviations: magnetic resonance imaging (MRI) and computed tomography (CT); DIPG, diffuse intrinsic pontine glioma; CSF, cerebro-spinal fluid; ONC201, small molecules which selectively targets Dopamine Receptor D2 (DRD2) and Caseinolytic peptidase P (ClpP); ERK, extracellular signal-regulated kinase; AKT, protein kinase B; ACVR1, activin A receptor type I; STAT3, signal transducer and activator of transcription 3; WEE1, WEE1 G2 checkpoint kinase; EGFR, epidermal growth factor receptor; VEGF, vascular endothelial growth factor; CAR-T, chimeric antigen receptor T-cell immunotherapy.

2. Methodology for literature search

The literature search was performed using Pubmed, Google Scholar, and Web of Science databases for articles published up to February 28, 2023, in English. The searching key words were set as "Diffuse intrinsic pontine glioma" OR "DIPG" OR "Diffuse midline glioma (DMG)" OR "H3K27M" OR "diagnosis" OR "therapy" OR "pediatric high-grad brain tumor" OR "cancer" OR "brain disease" OR "drug delivery" OR "extracellular vesicles" OR "exosomes".

The retrieved articles with duplicate titles, non-peer reviewed, or retracted were excluded initially. The selected articles included reviews, articles, books and documents, website, and clinical trials. Two investigators independently reviewed the list of retrieved articles and selected potentially relevant articles. The selected articles were focused on pediatric high-grade brain tumor, cancer diagnosis and therapy, brain disease, and extracellular vesicles. All clinical trials were searched from ClinicalTrials.gov, and only recruiting studies were included for further analysis. It must be admitted that our results were limited by the number of the selected articles and the understanding of this field.

3. Molecular mechanisms of DIPG

Epigenetic factors are known to drive cancer development and therapeutic response [42,43]. Therefore, the dysfunction of epigenetic molecules has been widely explored as potential targets for cancer treatment. DIPG, as a striking example of this theory, is one of the best-studied cancer diseases regulated by histone genes expression. In DIPG, there are more than 80% cases found to carry H3K27M mutation, located in H3.1 and H3.3 [44]. The H3K27M mutation refers to the replacement of residue 27 Lysine with Methionine, resulting in a global decrease of H3 trimethylation. This mutation induces the deactivation of polycomb repressive complex 2 (PRC2) methyltransferase, thus causing dysregulation of gene expression and further promoting glioma genesis [45,46]. Substantial research, driven by the molecular mechanistic implications of the H3K27M mutation, has focused on targeting the epigenetic molecules. Table 1 lists several remarkable efforts on exploring the molecular mechanisms and treatments for DIPG. Among these studies, advanced technologies, such as clustered regularly interspaced short palindromic repeat /CRISPR-associated protein 9 (CRISPR/Cas9) knockout screening, omics sequencing, and big data mining, played important roles in expanding and deepening our understanding of DIPG.

4. Traditional therapeutic strategies of DIPG

Generally, DIPG confirmation is based on symptoms, images, and biopsy/autopsy analysis (Fig. 1). Since surgical resection is not available most of the time, traditional therapies for DIPG, including radiotherapy and chemotherapy, will be discussed in this section.

4.1. Radiation therapy (RT)

Due to the high complexity of surgical resection and on-going development of chemotherapy, RT is still the standard of care for newly diagnosed DIPG patients. Therefore, a large quantity of research has been focused on analyzing improvements of RT on the OS of DIPG. Gallitto et al. systematically reviewed all available research in DIPG treatment with RT up to 2018 [2]. As one of the largest well-organized meta-analysis of RT for DIPG, it identified three types of definitive RT, namely upfront conventionally fractionated RT, hypofractionated RT, and hyperfractionated RT. The conventional RT mainly performed a total of 50–60 Gy with 1.8–2 Gy per dose, hypofractionated RT conducted with 3 Gy per dose and accumulated less than 45 Gy, whereas hyperfractionated RT required highest treatment dosage (more than 65 Gy altogether with 1 Gy per dose). However, none of the RT schemes significantly improved OS with a satisfied outcome (OS of 12.0, 10.2,

Table 1
Example of therapeutic vulnerabilities in DIPG.

Gene/ Protein	Drug	Mechanism	References
ACVR1, ACVR1 R206H, ACVR1 G328V	● VEGFR/RET/EGFR inhibitor vandetanib and mTOR/FKBP12 inhibitor everolimus ● ACVR1 inhibitor LDN212854 ● TβRI inhibitors	● Inhibit ALK2 enzyme activity ● Upregulates mesenchymal markers and activates STAT3 signaling ● Potential cross-talk between ACVR1 and TβRI pathways	[47–49]
PPM1D	● MDM2 inhibitor RG7388, AMG232, Nutlin-3 ● PPM1D inhibitor GSK2830371, NAMPT inhibitors	● P53 pathway ● DNA damage response ● Silence NAPRT gene	[50–52]
EZH2	● EZH2 inhibitors	● PRC2 subunit	[53,54]
BMI-1	● BMI-1 modulator PTC596 and ionizing radiation ● BMI-1 inhibitor PTC028 and BH3 mimetics	● Stimulates PRC1 E3 ligase activity by interacting and stabilizing the catalytic subunit RING1B ● PRC1 component ● Mitotic abnormalities associated	[55–57]
BET	● BET inhibitor JQ1 and CBP inhibitor ICG001 ● BET inhibitor JQ1 and EZH2 inhibitor EPZ6438	● Acetylation-dependent factors	[58–60]
RAS	● ERK5 inhibitors TG02 ● MYC inhibitor Omomyc	● RAS pathway ● ERK5 stabilize the proto-oncogene MYC	[61,62]
STAT3	● STAT3 inhibitor AG490 combined with radiation ● STAT3 pathway inhibitor WP1066	● A critical molecule for the differentiation of neural stem cells into astrocytes during neurodevelopment	[12,63]
B7-H3, CD276	● Antibody-based immunotherapy against B7-H3	● Immunoreactivity	[64,65]
WEE1	● WEE1 kinase inhibitor adavosertib combined with radiation	● WEE1 kinase pathway ● Control the G2 cell cycle checkpoint	[66,67]
PP2A, FGFR	● FGFR inhibitor ponatinib; PP2A inhibitor LB-100	● Dephosphorylation of PI3K/AKT and MAPK/ERK pathways.	[68,69]
LSD1, HDACs	● Corin, a bifunctional inhibitor of HDACs and LSD1	● Regulating gene expression and cellular differentiation ● Chromatin-modifying enzymes	[70,71]
DRD2	● ONC201, DRD2/3 antagonist	● G protein-coupled receptor ● P53-independent response ● Activation of ISR	[72–75]

Abbreviations: ACVR1, activin A receptor type I; VEGFR, vascular endothelial growth factor receptor; RET, ret proto-oncogene; EGFR, epidermal growth factor receptor; mTOR, mammalian target of rapamycin; FKBP12, FK506-binding protein 1 A; TβRI, transforming growth factor-β receptor type I; ALK2, activin receptor-like kinase-2; STAT3, signal transducer and activator of transcription 3; PPM1D, protein phosphatase 1D; MDM2, mouse double minute 2; P53, tumor protein P53; DNA, deoxyribonucleic acid; NAPRT, nicotinate phosphoribosyltransferase; EZH2, enhancer of zeste homolog 2; PRC2, polycomb repressive complex 2; BMI-1, B-lymphoma moloney murine leukemia virus insertion region-1; BH3, boron tri- hydride; PRC1, polycomb repressive complex 1; RING1B, really interesting new gene 1B; BET, bromodomain and extra-terminal domain; CBP: CREB-binding protein; RAS, rat sarcoma virus; ERK5, extracellular signal-regulated kinase 5; MYC, MYC proto-oncogene; B7-H3, B7 homolog 3 protein; CD276, cluster of differentiation 276; WEE1, WEE1 G2 checkpoint

kinase; PP2A, protein phosphatase 2; FGFR, fibroblast growth factor receptor; PI3K, phosphatidylinositol 3-kinase; AKT, protein kinase B; MAPK, mitogen-activated protein kinase; LSD1, lysine specific demethylase 1; HDACs, histone deacetylases; DRD2, dopamine receptor D2; ISR, integrated stress response.

and 7.9 months respectively), not to mention the RT induced side effects and radio-resistance. Park et al. compared conventional fractionated RT and hypofractionated RT on the treatment of DIPG patients, and found no difference between these two treatments [76]. Izzuddeen et al. reported that combination of hypofractionated RT and temozolomide (TMZ) treatment could not improve OS, and even displayed higher hematological toxicity [77]. Therefore, although RT is a standard care for DIPG, there is still an urgent need to develop other therapeutic approaches.

4.2. Chemotherapy

Chemotherapy, as a traditional adjuvant therapy, is also used in the treatment of DIPG. As the first-line chemotherapeutic agent for newly diagnosed glioblastoma (GBM), TMZ is still under consideration in combination with multiple treatment modalities to treat DIPG. In 2019, Chua et al. discussed the utilization of TMZ for DIPG therapy in a review [78], and they found no evidence to prove DIPG patients obtain benefits from TMZ treatment. Over the past decades, failure of the traditional cytotoxic chemotherapy for DIPG could be explained by numerous reasons [8,79,80]: (1) the presence of BBB and BBTB; (2) more compact structure of the brainstem; and (3) difficulty in evaluating drug efficacy due to the anatomical location of the tumor. Although some therapeutics showed nonsignificant results [81], the hope for DIPG treatment is still alive. The hope is based on the improvement of current technologies and the development of more systematic, rational clinical trials.

Currently, seven of the forty-three DIPG-recruiting clinical trials are using TMZ as one of the treatments [82], which will be completed between 2022 and 2027 (NCT03396575, NCT04049669, NCT03243461, NCT04239092, NCT01837862, NCT03709680, and NCT04238819). In these clinical trials, TMZ has been combined with: (1) radiotherapy, (2) chemotherapy, (3) immunotherapy such as antibodies or dendritic cell vaccines, (4) novel inhibitors, and (5) other drugs (Anti-epilepsy drug valproic acid and anti-parasitic drug mebendazole).

5. Novel therapies for DIPG

With the development of technologies and understanding of cancer, more and more effective clinical treatments have been applied to the therapy of malignant tumors. It is undoubtedly a great challenge and opportunity for DIPG without significant effective therapeutics. In recent years, a series of therapeutics and technologies such as chimeric antigen receptor T-cell (CAR-T) immunotherapy, small molecule inhibitors of critical signaling pathways, and EVs have been gradually developed for the treatment of DIPG.

5.1. Immunotherapy

Glioma is a typical cold tumor which has a low immune response, and DIPG is extremely “cold” due to the occurrence location of the tumor cells. Therefore, immunotherapies failed that much due to the suppressive immune microenvironment. Under this circumstance, chemotherapy can be applied to either enhance the immune response or combine with immunotherapy. It is highlighted that Bailey et al. discovered the bifunction of the lysin-specific demethylase 1 (LSD1) in controlling cell death and immune responses, and the LSD1 inhibitor enhances NK cell cytotoxicity in pediatric high-grade glioma [71]. Another encouraging result is discovered by Mackall’s and Monje’s lab. They showed that Anti-GD2 CAR-T cell administration was a possible way to treat H3K27M mutant diffuse midline gliomas [83,84]. What we can learn from this study is, not only the well tolerance and efficacy of CAR-T cell against brain tumor, but also that the disialoganglioside GD2 can be a treatment target for H3K27M mutant gliomas. Therefore, future studies can develop more specific therapeutic strategies on the basis of this novel target.

5.2. Inhibitors

Thanks to the application of advanced screening strategies, modern chemotherapy (like small molecule inhibitors) developed to be more attractive and more specific to target vulnerabilities of DIPG (Table 1). Remarkably, Carvalho et al. used artificial intelligence (AI) approach to discovered that vandetanib and everolimus combination is a potential therapeutic strategy for *ACVR1*-mutant DIPG [47]. Xu et al. identified that RG7388, a mouse double minute 2 (MDM2) inhibitor, suppresses the proliferation of tumor protein P53 (*TP53)* wild-type/*PPM1D* mutant DIPG cells lines in a p53-dependent manner [50]. A selective DRD2/3 antagonist ONC201 has been shown to cross BBB efficiently and to exhibit significant efficacy in H3K27M mutant DIPG patients [72,73,85,86]. Currently, two ongoing phase I/II clinical trials of ONC201 (NCT03416530 and NCT05009992) are recruiting patients with H3K27M mutant DIPG. Meanwhile, the Diffuse Midline Glioma-Adaptive Combinatory Trial (DMG-ACT) has started in Europe, Australia, and the United States of America.

Additionally, these small inhibitors when used in combination with RT or other strategies displayed promising results. For example, utilization of histone demethylase inhibitor GSK-J4 showed enhanced effect when combined with RT for DIPG cells [87,88]. Inhibition of WEE1 kinase by MK-1775 could be combined with radiotherapy for the treatment of DIPG [67]. A phase I/II study of cyclin D-CDK4/6 inhibitor ribociclib treatment following conventional RT led to increase of 1-year and median OS to 89 % and 16.1, respectively [89].

5.3. Nanotherapy

Nanotechnology is no doubt to be a revolutionary technology that entered our daily life and medical application, namely nanotherapy [90]. As a significant hallmark for combating cancer, nanotherapy has two major advantages over other treatments: specific tissue uptake and adequate biodistribution. Nanoparticles (NPs) such as EVs, liposomes, biomaterial polymeric systems, gold NPs, magnetic NPs, carbon nanotubes, etc. all displayed high efficiency in crossing various biological barriers. Surface modification plus natural properties enable NPs to load sufficient drugs and delivery them to the targeted location. The application of nanotherapy in GBM has been explored for years but very few are specific to DIPG [91]. More recently, Ung et al. showed the possibility of using gold NPs to deliver doxorubicin across DIPG spheroids [92]. Shargh et al. established a nanoparticle-based potent N (3)-propargyl analog (N3P) system, illustrating the high stability and efficiency of the NPs drug against TMZ-resistant DIPG cells in vitro and in vivo [93]. The excellent performance in a series of CNS neoplasms all shows that nanotherapy has great prospects in the treatment of brain diseases. As one of the best representative nanotherapy, due to the unique biological characteristics, there will be huge scope for the development of EVs in cancers.

6. Extracellular vesicles in cancers

With Profs. James E. Rothman, Randy W. Schekman, and Thomas C. Südhof won the 2013 Nobel Prize in Physiology or Medicine, lots of researchers focus their research field on these nanoscale vesicles over the last decade. EVs carry abundant intracellular substances and membrane proteins and participate in almost all biological processes. Based on the natural characteristics of EVs, mechanism exploration and clinical translation have become hot spots in recent years.

6.1. General information

EVs are membrane-encapsulated vesicles released by almost all cell types. EVs are also present at very high concentrations, ranging from millions to billions/ml, in all body fluids [21,22], including milk, saliva, tears, vaginal lavage, semen, and blood [40,41,94–109]. A phospholipid membrane protects its bioactive cargoes (e.g. proteins, lipids, and RNAs) from degradation in the intra- and extra-cellular environment. The direct interaction between EVs and cells has become the basis of a short- or long-distance intercellular communication mechanism whereby EVs trigger a cellular response in host/recipient cells. According to the guidelines of the international society of extracellular vesicles (ISEV), EVs can be classified based on several rules: (a) physical characteristics (characterized by nanoparticle tracking analysis or electron microscopy) such as size and density, or (b) biochemical composition (detected by western blot or flow cytometry) such as tetraspanin + /- EVs, or (c) cell origins or conditions [110]. Although EVs were firstly defined as “trash” eliminated by cells, today, EVs are important research, diagnostic, therapeutic, or drug delivery tools [34,101,102,105,111].

The main challenge of EVs application is the isolation of EVs from a variety of body fluids and tissues, and the recovery of the biological function of obtained EVs with good purity. As the “gold standard” of EV isolation, ultracentrifugation becomes the most popular method with or without combining with other methods. However, it must be admitted that EVs are such heterogenous groups that ultracentrifugation cannot simply separate them from each other. Therefore, efforts never stopped to explore faster, cleaner, and more specific techniques for EV subpopulation isolation, such as immune-captured method [112], asymmetric flow field-flow fractionation (AF4) [113], nano-flow cytometry coupled with high-resolution microscopy [114], etc. Among these methods, the size exclusion chromatography (SEC) based approach, namely novel particle purification liquid chromatography (PPLC), displayed significant improvements in the identification, tandem purification, and characterization of both biological and synthetic nano-scaled membrane vesicles [40,98]. It is plausible that the discovery of these novel methods will speed up the application of EVs in biomedical sciences. (Fig. 2).

6.2. EVs as biomarker for diagnosis

The primary medical application of EVs is to serve as biomarkers for early and accurate diagnosis. Biomarkers in EVs should be detectable, measurable, and associated with specific disease significantly.

In cancers, for example, the unique tumor-derived molecules, including nucleic acids, proteins, phospholipids, saccharides, and metabolites, all have potential to be biomarkers for diagnosis [115]. Melo et al. distinguished early pancreatic cancer from benign pancreatic

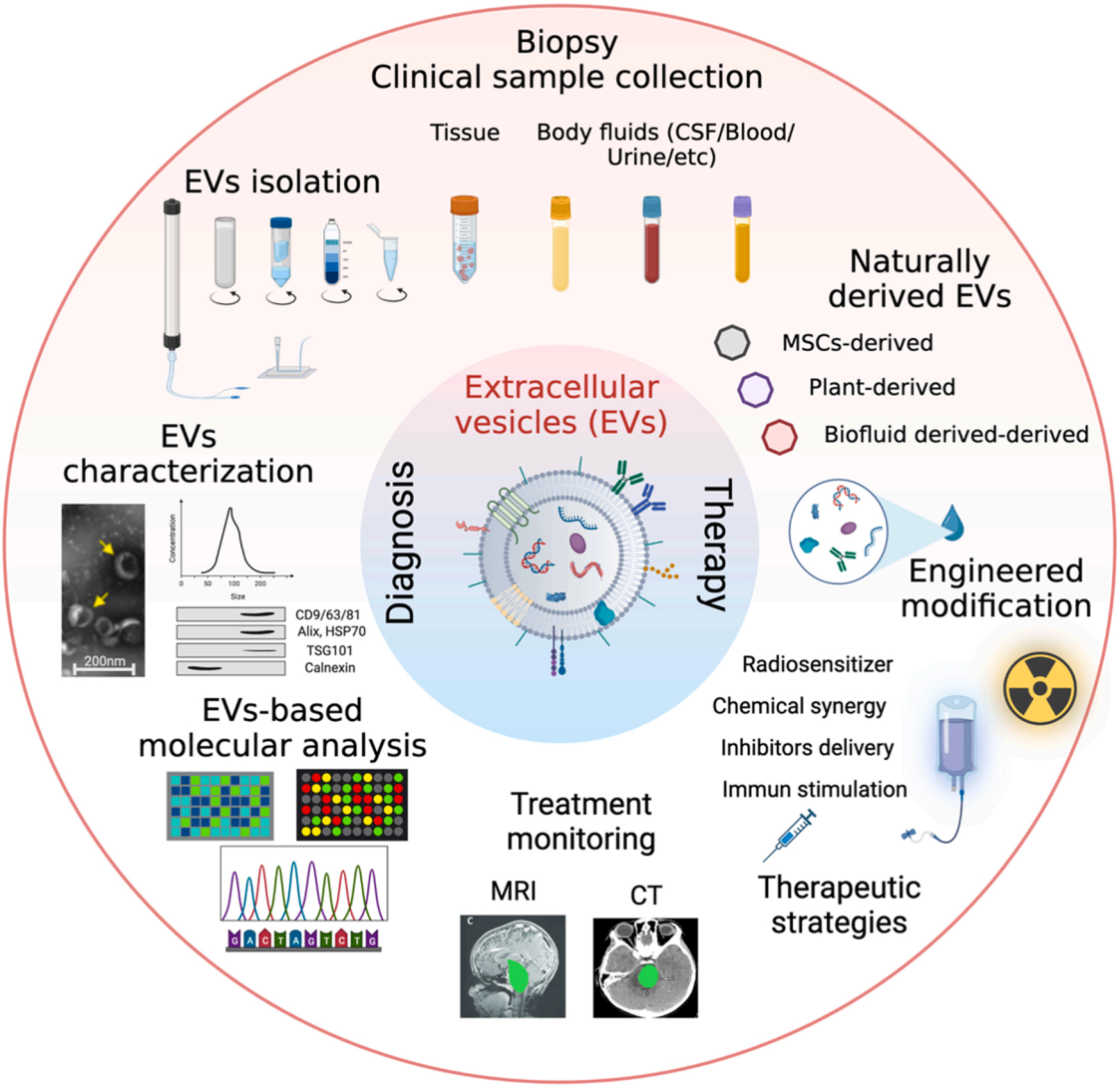

Fig. 2. The application of EVs in cancers. The transmission electron microscopy (TEM) image of EVs is recreated based on Lyu et al. [104]. Abbreviations: magnetic resonance imaging (MRI) and computed tomography (CT); CSF, cerebro-spinal fluid; MSCs: mesenchymal stromal cells.

diseases through a non-invasive test, which utilized Glypican-1 (GPC1) as biomarker for pancreatic cancerous circulating extracellular vesicles [116]. Independently, other labs found GPC1 are enriched in breast and colon cancer derived EVs, thus can be applied to detect early cancer [117,118]. In both glioma and serum EVs, Kang group identified PTRF/Cavin 1 as potential biomarkers [119].

Through advanced PPLC isolation technique, Alvarez et al. separated and characterized blood plasma derived EVs (BEVs) from 17 breast cancer patients that received neoadjuvant chemotherapy [95]. They found eight proteins were enriched in BEVs of nonpathological complete responders and validated through western blot and functional assay. This study not only highlighted the feasible utilization of PPLC-based EV separation technology, but also showed the well-preserved capability of EVs, implying BEVs can be used to predict response to cancer treatment.

Except protein, nucleic acid, especially RNA, is another remarkable sign for early diagnosis of diseases. Since most packed RNAs are less than 200 nucleotides, messenger RNA (mRNA) and microRNA (miRNA) are the most frequently detected in EVs and reported as novel biomarkers [103,120]. EVs also shelter these RNAs from enzymatic degradation, thus conserving their functions[103]. Wu et al. retrieved most studies before 2020 on the utilization of EV miRNAs from different body fluids for the diagnosis of lung cancer, concluded that the full role of EV-miRNA still require further investigation before applying for clinical application [121]. Wang et al. reviewed circulating EV-miRNAs as biomarkers for the neurodegenerative diseases (NDDs), including Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD), etc [122]. Their studies not only highlighted the potential of EV-miRNAs as biomarker, but also highlighted the need for further research and development in this area, including robust EVs extraction techniques and a deeper understanding about the relationship of EV-miRNAs and NDDs.

Circular RNAs (circRNAs), in addition, have been identified as pioneer biomarker for cancer diagnosis recently [123]. Consequently, EV-circRNAs also appeared to be a novel frontier as a biomarker for various diseases [124]. In 2019, the United States Food and Drug Administration (FDA) granted the first exosome-based liquid biopsy test, namely the ExoDx Prostate IntelliScore (EPI) Test, for a Breakthrough Device Designation Award [125]. Therefore, EV-based diagnosis has great market potential and deserve to be further explored.

6.3. EVs as vehicle for drug delivery

Besides carrying biomarkers for diagnosis, EVs may serve as a vehicle for drug delivery, since the lipid bilayer vesicle structure provides natural holding sites for both hydrophobic and hydrophilic molecules. Unlike EVs for diagnosis, therapeutic EVs needs to be purified, collected, modified, enriched, safely administered to the patient, and play specific roles in the target cells. Although some reports showed that EVs may not be stable in circulation [126,127], it seems due to various source of EV collection [128]. Therefore, it is important to understand the unique features of EVs that are suitable for drug delivery.

To develop an EV based therapeutic, cellular source is the first consideration, and thus mesenchymal stromal cells (MSCs) derived EVs are developed as potential candidate. This is because MSCs are found to participate in cancerous processes, including regulation of initiation, development, progression, and metastasis [129]. It is now clear that MSCs play their roles in a paracrine manner, thus MSCs-derived EVs standout during this process [130]. MSCs also possess tumor-homing properties, and MSCs-derived EVs may serve as drug delivery vehicles for cancer treatment.

Another advantage is that EVs have the ability to cross biological barriers. BBB as the major biological barrier in the brain, helps to protect the brain from threats from exogenous environment. Although the BBB has been investigated extensively, the mechanism of BBB penetration is still not clear, thus leading BBB the major obstacle for brain therapy [131]. As a result, the majority of brain medicinal design must consider BBB crossing efficiency. In the location of brain tumors, blood vessels grow into tumor tissue and retain the properties of BBB, which becomes the BBTB. The permeability of BBTB is easier than BBB due to the fast formation of blood vessels, but therapeutic medicines of brain cancer still have to overcome this obstacle [132]. Niu et al. developed a novel biomodified EVs that originated from natural grapefruit, displaying efficient BBB/BBTB crossing ability of modified EVs and anti-glioma potential [133]. This study not only showed the BBB/BBTB bypassing property of modified EVs, but also highlighted that naturally derived EVs may be a good delivery platform for glioma therapeutics. However, the safety of exogenous EVs should be analyzed and confirmed before clinical application.

It is known that EVs may carry tumorigenic major histocompatibility complex class (MHC)-II [134,135], which is responsible for stimulating CD4 + and CD8 + T cells, and from a medicinal aspect, this property of EVs may be applied to stimulate immune cells for cancer treatment. Kalluri et al. discussed the role of EVs in immune responses [27,136], emphasizing the potential of EV in triggering immune signaling pathway, manipulating gene expression, and activating relative immune cells. Xu et al. systematically reviewed the potential utilization of EVs in cancer immunotherapy [137], concluding that EVs may be applied as either cancer vaccines or antigen/drug vehicles.

6.4. Engineered EVs for targeted therapy

In order to obtain ideal therapeutic EVs, appropriate incorporations and modifications, including bioactivity, drug loading efficiency, and targeting efficiency need to be considered. General modification strategies can be classified into pre-loading method (endogenous method) and post-loading method (exogenous method) [138]. (Fig. 3).

For pre-loading method, MSCs are usually the first choice to obtain therapeutic EVs as mentioned above. Other natural cell-derived EVs, such as dendritic cell-derived EVs [134,139], tumor cell-derived EVs [140], natural killer (NK) cell-derived EVs [141–143], macrophage-derived EVs [144,145], body fluid-derived EVs [146,147], and plant-derived EVs (such as grapefruit, ginger, citrus, etc. [133, 148–150]), also showed potential as novel therapeutic agents. Pre-loading strategy is less tedious, but it is difficult to control the drug bioactivity, since donor cells can react to the loaded drugs and produce a series of metabolites. While natural EVs are highly heterogeneous even from the same source of cell line, further separation and enrichment of EVs subpopulation will increase the bioactivity of therapeutic EVs [151]. Drug loading efficiency analysis also needs to be standardized to be efficient. Hendrix lab established a gag-fluorescence recombinant EV, which can be tracked easily and distinguished from sample EV, in order to calibrate instrument and normalize data [152]. Silva et al. reported a workflow to quantify the loading efficiency of protein cargos into engineered EVs [153]. Firstly, they collected engineered EVs with sorting proteins fused to green fluorescent protein (GFP), confirming the success of loading with western blot. But Nanoflow cytometry, ExoView Tetraspanin chips, and single-molecule localization microscopy assays revealed GFP loading in less than half of EVs, reflecting the heterogeneity of EVs. This study not only introduced a couple of techniques that can be used to quantify protein drug loading efficiency but also pointed out the top candidate subpopulation for loading GFPs into EVs.

Genetic cargos can be loaded into donor cells through transfection, and these cargos can be DNA plasmid, miRNAs, mRNAs, long non-coding RNA (lncRNA), etc [154–156]. Transient CRISPR-based delivery offers high gene editing efficiency, low off-target effects, and low immune responses. EVs are proved to be suitable for Cas9 ribonucleoprotein transportation [157,158]. Yao et al. developed a novel approach to specifically enrich Cas9 and adenine base editor ribonucleoprotein into EVs, keeping the genome editing activity at the mean time [159]. However, it is yet to be determined the safety and off-target concerns about EV-based delivery of CRISPR/Cas9 system. Gee et al. demonstrated an efficient delivery system of CRISPP/Cas9 and sgRNA into EVs

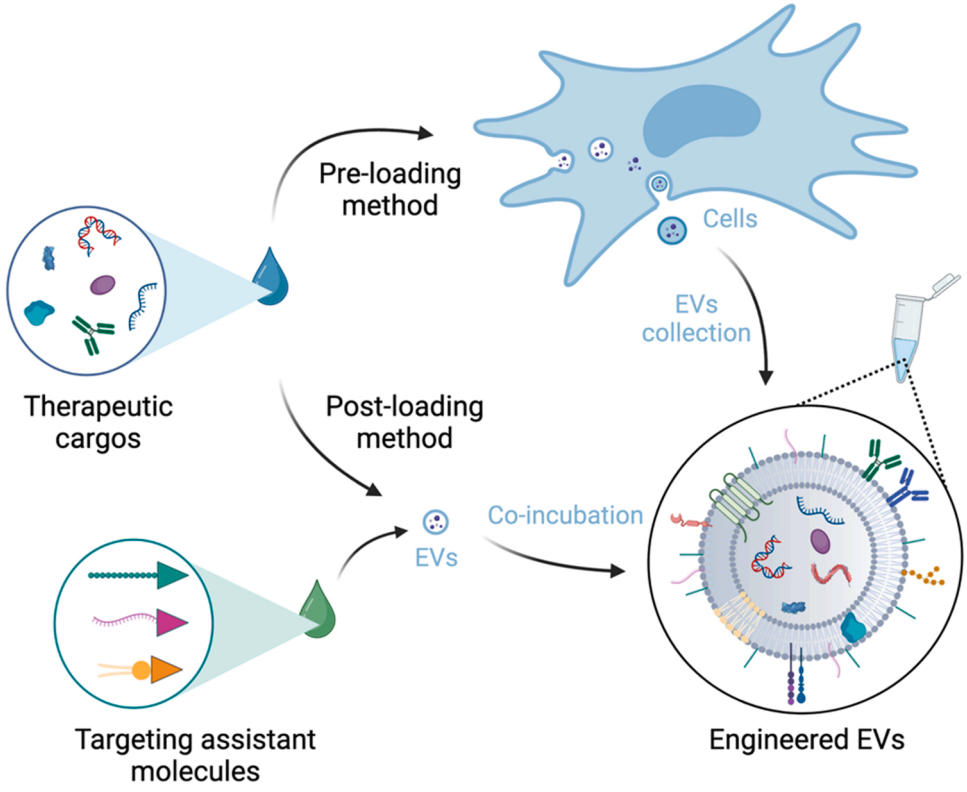

Fig. 3. Strategies for engineered EVs production.

[158]. Their method not only showed the high efficiency of EV-based genome edition, but also demonstrated the potential of using EVs for in vivo therapy.

For post-loading method, EVs will be collected first and mixed with different drugs at conditions empirically determined. Drugs will incorporate with EVs either on the surface or enter the EVs. The hydrophobicity and electrostatic character of the drugs are the key factors that determine the loading efficiency. Lipid composition of EVs also appears to be important [160]. This is understandable since the biophysical properties of EVs, such as membrane thickness, curvature, occupation area, etc. depend on the lipid types, which in turn affects drug loading efficiency [161]. Thus, extra techniques that assist drug loading into EVs are mainly considered to improve the hydrophobicity of drugs or increase membrane permeability temporarily. Electroporation has been shown to form transit pores on the membrane surface of EVs, thus creating temporal entrances for cargo drugs [162–164]. Inspired by the liposomal active loading mechanisms, Chen et al. developed a sonication and extrusion assisted active loading method (SEAL), in order to encapsulate drugs into EVs effectively and stably [165]. In this method, sonication provides temporary permeability of EV membranes and extrusion homogenized the particle size of EVs. However, EVs are not the same as liposomes: pores may not seal at certain subpopulations and induce EVs aggregation, thereby disrupting the integrity of the EVs and their drug loading capacity.

To increase the accuracy of targeted delivery, modifications on EVs surface are often required. Proteins and peptides are two common tools that can assist in the transportation of EVs to their targets. Chen et al. constructed engineered EVs overexpressing programmed death 1 (PD-1), which efficiently inhibited the proliferation of programmed death 1 ligand 1 (PD-L1) overexpressing cancer cells and induced the apoptosis of the cancer cells [166]. Jia et al. established glioma-targeting EVs by conjugating neuropilin-1-targeted peptide (RGERPPR, RGE) through click chemistry [167]. Tian et al. applied targeting peptide c(RGDyK) to the surface of DBCO-modified EVs through click chemistry as well, to deliver curcumin for cerebral ischemia therapy [168]. Except for these covalent modifications, non-covalent incorporation techniques are also attractive [169]. Generally, cancer cells are more negatively charged than normal cells, which provides an opportunity for targeting therapy. Researchers produced EVs with a positive charged surface by anchoring multiple molecules: cationic amphiphilic macromolecule ε-polylysine-polyethylene-distearyl phosphatidylethanolamine (PPD) [170], cationized mannan [171], cationic pullulan [172], positively charged lipid [173], etc. Compare to these options, peptides are a better choice since the peptide sequence can be changed variously to improve charges, hydrophobicity, homing probability, and binding affinity [174–176]. Peptide sequences are also much shorter than protein sequences, making production easier and cheaper [177]. Therefore, peptide-engineered EVs deserve to be studied extensively to target tumor cells more accurately and efficiently.

7. Potential use of EVs in DIPG

7.1. EVs as diagnosis methods

It is undoubtedly certain that early and accurate diagnosis is one of the primary hurdles for the confirmation of DIPG. General diagnosis for DIPG is usually delayed 2–3 months after the appearance of clinical symptoms showed up [8]. The first option for doctor is to prescribe computerized tomography (CT) and magnetic resonance imaging (MRI) to obtain the images of patients. To further assist imaging diagnosis, a stereotactic brainstem biopsy will be taken for tissue analysis. While stereotactic biopsy is not widely accepted, biofluid biopsy (such as cerebrospinal fluid (CSF), blood plasma, urine, etc.) can provide abundant information to assist diagnosis as well. Garnier et al. demonstrated distinct molecular subtypes of biofluid EVs from brain tumor patients through proteomics analysis [178]. Stallard et al. reported that the *H3F3A* K27M copies in pediatric DIPG CSF has statistical relationship with imaging results [179]. These two studies all demonstrated the

clinic value of biofluid biopsy. Since the presence of functional EVs in the CSF of GBM patients has been reported before [180,181], it is reasonable to hypothesis DIPG CSF will also contain EVs carrying important information molecules. Beyond that, Magaña et al. evaluated EVs from DIPG-derived cell lines through high-throughput next-generation sequencing [182]. They found that DIPG EVs carry a variety of non-coding RNA cargos, highlighting potential miRNAs that can be used for diagnosis. While multiple studies have proved the presence of miRNA in GBM EVs as biomarker [183–185], further validation of DIPG biomarkers in patients is still needed.

7.2. EVs as treatment monitoring approaches

Given that tumor-derived EVs carry a bunch of important cargo from parental tumor cells, it is attractive to utilize this not only for early diagnosis but also for monitoring treatment responses. For example, the protein component of tumor-derived EVs was reported to change along with cancer therapy [186], and the miRNA profile of serum EVs was demonstrated to be associated with esophageal squamous cell carcinoma recurrence [187]. With longitudinal monitoring of the plasma EV-based DNA of the patients under treatment, Bernard et al. showed that the circulating nucleic acids from EVs are associated with the outcomes of pancreatic cancer patients [188]. While EVs carry important information from the targeted cells, they can be used to detect resistance-associated molecules. Wei et al. illustrated the exosomal miR-222–3p as a principal regulator of gemcitabine resistance, cell proliferation, and malignant properties via targeting suppressor of cytokine signaling 3 (SOCS3) [189]. Other neural diseases, such as AD [190,191], PD [192], HD [193], ischemic and hemorrhagic stroke [194], and drug-addictive issues [104,194], may also employ EVs for monitoring the development of treatment. In addition, EVs engineered with fluorescence or other detective molecules may also serve as tracking tools in vivo [195,196]. Therefore, the application of EVs for DIPG treatment monitoring deserves to be further investigated.

7.3. EVs as therapeutic strategies

The presence of BBB and brainstem structure block most of the drugs, including small molecule compounds for radiosensitizer and antibody protein for immunotherapy, thereby limiting the efficacy of therapeutics. But as mentioned before, it was shown that fluorescently labeled EVs may deliver anticancer drugs across the BBB in a zebrafish model [33]. Other studies also showed that engineered EVs from various sources may cross the BBB [32,164,197,198]. Kang group successfully utilized blood EVs for GBM treatment in vitro and in vivo, their study showed that modified EVs can cross BBB and target tumor cells [199–201]. Sun group also developed dopamine-loaded blood EVs that can cross BBB and improve the treatment of PD [202]. In addition, EVs can work as the communicator between neuron-neuron and neuron-glia. This brings the possibility of delivering drugs through EVs to local neurons. Xia et al. pointed out the neurotransmitter role of EVs through a systematic review, discussing the biological function of EVs in regulating neurotransmission [203]. Yuan et al. reported that macrophage-derived EVs can supply proteins to the inflamed brain [204]. Although there are no current clinical trials for brain cancer, there are more than 11% recruiting clinical trials on EVs application focusing on brain diseases. Therefore, the application of engineered EVs in DIPG diagnosis and therapy still requires extensive advanced research and preclinical validation.

8. Conclusion remarks

Although pediatric high-grade brain tumor, DIPG, is still a severe disease in children, it is evident that the current understanding of DIPG has been deeper than the past decades. H3K27M and relevant mutations in DIPG patients induce global loss of histone trimethylation and further influence the downstream epigenetic function of cells. Multiple molecular targets, thereby, have been developed for DIPG therapy.

In this context, this study has raised important questions about the diagnosis and treatment of the pediatric high-grade brain tumor, DIPG. Novel technologies such as immunotherapy and small molecule inhibitors started to exhibit encouraging results. Therefore, the prognosis and OS of DIPG will certainly be promoted when more researchers start to focus on this disease through advanced therapeutic strategies. EVs, as one of the most promising options, deserve to be paid more attention, since the unique crossing ability, low immunogenicity, and high cargo loading properties enable EVs to transfer multiple drugs into the tumor region safely and effectively. It is also certain that EVs research field is still facing a couple of challenges. For example, the search for DIPG EV biomarkers through liquid biopsy, improving the target accuracy, and maximizing the drug loading efficiency of EVs all remain to be determined. The combination of EVs with immunotherapy and chemotherapy is also promising and attractive.

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CRediT authorship contribution statement

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Declaration of Competing Interest

We are writing to declare that we have no known competing financial interests or personal relationships that could have appeared to fluence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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