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Overview of Retroviral Transduction of HSV-TK Gene in the Gene Suicide Therapy for Glioblastoma Multiform

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Abstract. Glioblastoma Multiform (GB) is the most malignant tumor of the brain. The treatment of GBM is a challenging task worldwide, and the prognosis of GBM remains poor, thus demanding the research of new and improved therapies. One new approach in treating GBM is through the transduction of the Herpes simplex Virus Thymidine Kinase (HSV-TK) gene, followed by ganciclovir (GCV) administration. During GBM treatment, the HSV-TK gene is first delivered into the host by retroviruses, one of the most commonly used vectors in said therapy due to their ability to selectively modify actively dividing cancer cells. Following the expression of HSV-TK, the HSV-TK enzyme catalyzes the phosphorylation of GCV into a cytotoxic metabolite, leading to the apoptosis of the target cell. Moreover, a phenomenon observed in cancer cells during gene suicide therapy named the"bystander effect" further enhanced CV cytotoxicity. Said effect enables the transfer of GCV-3P to surrounding cancer cells without HSV-TK transduction and expression, elevating cytotoxic effects throughout the tumor. However, due to the heterogeneous nature of GBM, relapse of the tumor is almost certain after HSV-TK therapy. Additionally, tumor-associated macrophages in the tumor microenvironment (TME) of GBM further enhance treatment difficulty. Further research should go towards understanding the TME of GBM to personalize better and design more effective treatments.

Keywords: HSV-TK, Retroviral Vector, Glioblastoma Multiform, Ganciclovir, Bystander Effect.

1. Introduction

Glioblastoma Multiforme (GBM), a grade 4 glioma, is the most malignant yet common central nervous system tumor originating in the brain [1]. No definite risk factors and prevention strategies for GBM have been identified; however, some debate that possible risk factors include prior exposure to radiotherapy, cell phone use, and specific inherited syndromes [2]. With proper therapeutic procedures, the 5-year survival rate for GBM is between 5% and 10%, and a median life expectancy of 14 months. In contrast, GBM can be fatal in around 3-4 months without proper intervention [1]. The surgical resection of GBM tumors is usually suggested and performed in patients when possible, allowing pathologists to confirm the GBM diagnosis; however, the highly malignant nature of GBM results in an almost 100% relapse rate in patients [2]. Despite advancements in medicine and the constant discovery of new cancer therapies, no effective treatment for GBM has been found thus far [3].

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The first gene suicide therapy, through the transduction of the Herpes Simplex Virus Thymidine Kinase (HSV-TK) gene, was formulated in the 1990s and has since become one of the most researched and experimented gene therapies for cancer. The HSV-TK gene, as its name suggests, is found in the herpesvirus genome and expresses an enzyme in the family of thymidine kinases [4]. Thymidine Kinases (TKs) are phosphotransferases that catalyze the transfer of a phosphate group from adenosine triphosphate (ATP) to thymidine to form adenosine diphosphate and thymidine monophosphate, an integral process to DNA synthesis [5]. Human cellular TK is strictly specific to the phosphorylation of thymidine. Conversely, HSV-TK has a broader specificity with the ability to phosphorylate purine and pyrimidine analogs in addition to thymidine. The broad specificity of HSV-TK is the target of several antivirals for treating herpes, such as using purine analogs as HSV-TK competitive inhibitors to prevent the phosphorylation of thymidine and to synthesize cytotoxic metabolites in infected cells [6]. The discovery of the HSV-TK gene paved the way for the invention of the aforementioned gene suicide therapy. In said therapy, the transduction of the HSV-TK gene into the resection cavity of GBM by the use of a retroviral vector is followed by the intravenous administration of ganciclovir (GCV), a guanosine nucleoside analog antiviral [5, 6]. The expression of HSV-TK in target GBM cells initiates the metabolic pathway of GCV. The prodrug GCV is metabolized into a cytotoxic intermediate–ganciclovir triphosphate (GCV-3P), inhibiting DNA replication and inducing apoptosis in GBM cells [5].

This paper will first provide an overview of the use of retroviral vectors in HSV-TK gene suicide therapy, the mechanisms and effects of GCV metabolism in GBM, and potential limitations in current treatment approaches.

2. Retroviral Vector

2.1. Retrovirus and Adenovirus Replication Cycle

Retroviruses are RNA viruses with the enzymes integrase and reverse transcriptase [8]. One of the main differences between a retroviral vector and an adenoviral vector lies within their respective lifecycles. Most adenoviruses replicate by the lytic pathway, and most retroviruses replicate by the lysogenic cycle [9, 10]. This difference offers theoretical advantages and drawbacks in gene suicide therapy for GBM, which will be further discussed.

In both the lifecycle of adenoviruses and retroviruses, entry into a host cell must first be achieved. Retroviruses attach to the host cell, initiating the fusion between the retrovirus lipid envelope and the host cell's phospholipid surface membrane and allowing the viral core to enter the host cell [11]. Following entry, the enzyme reverse transcriptase, also known as synthesizes a double-stranded DNA from a single-stranded RNA in the retrovirus. Since reverse transcriptase uses an RNA strand as a template for DNA synthesis, it is also known as RNA-dependent DNA polymerase [12]. The newly synthesized DNA strand then enters the host cell nucleus for integration into the host chromosome, catalyzed by the enzyme integrase, which cleaves the host genome to allow the insertion of viral DNA [13, 14]. In particular, the DNA strand only enters the target cell nucleus during mitosis, as the nuclear membrane has broken down [13]. Notably, Retroviruses have two strands of RNA in their capsids. Although not perfectly established, it has been theorized that the double-stranded RNA increases the probability of reverse transcription and DNA integration, as one RNA strand will still be available following a strand break [15]. Following successful integration, the viral DNA will undergo transcription and translation to synthesize viral proteins, which can bud off from the host cell to infect other cells [13]. Unlike retroviruses, adenoviruses do not integrate their DNA into the host cell genome, as they do not have the enzyme integrase. Adenovirus DNA enters the nucleus after the fusion of the virus and host cell, utilizing host cell transcription machinery directly to synthesize viral primary transcripts. The primary transcripts are subsequently spliced and are translated into viral proteins using host ribosomes [16]. The synthesized viral proteins assemble into viral components, released from the host cell following host cell lysis induced by the virus [17].

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2.2. Discussion of Retroviral vector benefits and drawbacks

The main benefits of using a retrovirus vector for HSV-TK gene suicide therapy for GBM are due to the permanent DNA integration of retroviruses. Retroviruses do not result in the lysis of GBM cells with HSV-TK transduction due to their lysogenic nature, allowing the HSV-TK gene to be transferred throughout the tumor while remaining undetected by the host's immune system [18]. With adenoviruses, the lysis of tumor cells can release pathogen-associated molecular patterns, stimulating the immune system and halting the transduction of the HSV-TK gene [19]. Moreover, due to the integration of retroviral DNA, the HSV-TK gene delivered by a retroviral vector will only replicate when the host cell divides [15]. Thus, another advantage of using retroviral vectors is their specificity in targeting and infecting dividing cancer cells [20, 21]. As a result of the inability of fully differentiated neurons to undergo mitosis, the cytotoxicity of HSV-TK therapy toward healthy brain tissue will be reduced with retroviral vectors [21].

Despite such advantages, hypothetical drawbacks of using a retroviral vector for HSV-TK therapy for GBM also exist. Retroviruses' dependence on host cell division for replication dramatically reduces their efficiency in gene transduction compared to other vectors. For example, adenoviruses are typically more aggressive than retroviruses, infecting both dividing and non-dividing cells, and can penetrate and deliver genes more efficiently to target regions [9, 22]. As a result of retroviruses' poor ability to spread throughout the tumor, several clinical trials reported that adenovirus-delivered HSV-TK therapy for GBM had more than double the survival time compared to those mediated by retroviral vectors [23]. The integration of retroviral DNA can also lead to further oncogenic activation due to insertional mutagenesis, which can further promote GBM proliferation or cause other cancers [9].

2.3. Making Retroviral Vectors

Before retroviruses can be used as vectors, their genomes must be modified to deliver the target gene and decrease infectivity. Retrovirus genomes typically comprise gag, pol, pro, and env genes and small non-coding RNA sequences. The gag gene encodes a structural polyprotein integral in the capsid and viral matrix assembly [24, 25]. The pol gene encodes the enzymes RT and integrase [24]. The pro gene encodes viral proteases, and the env gene encodes glycoproteins on the viral envelope [25]. The gag, pro, and pol genes are typically expressed as fusion polyproteins gag-pro-pol [15]. The expressed protease in this fusion polyprotein will self-cleave to form gag, pro, and pol proteins [25]. Following self-cleavage, the protease cleaves pol to create RT and integrase enzymes [24]. The gag protein will also be cleaved five more times to form 6 fragments for structural assembly [26]. The gag, pro, pol, and env genes are removed to reduce infectivity when preparing retroviral vectors for HSV-TK.

Following removal, the therapeutic cassette replaces These removed genes, containing the HSV-TK gene and respective promotors and enhancers [24]. However, if the gag, pro, pol, and env genes are removed from a retrovirus, the virus loses its ability to integrate the HSV-TK gene into the GBM cell genome in therapy. To enable retroviral gene integration, these necessary proteins are provided to the modified vectors through packaging cell lines [24]. In this process, plasmids encoding for gag, pro, pol, and env proteins are first stably integrated into the genome of packaging cells [25]. The HSV-TK gene therapeutic cassette is introduced into the packaging cells separately without gene expression. The assembled retroviral particles containing the HSV-TK gene and the necessary viral proteins are then collected and purified from the packaging cells [27, 28]. After retrovirus packaging, the produced viral particles can integrate their genome during transduction stably but cannot create new viral particles directly [28].

3. HSV-TK Mechanism

3.1. HSV-TK Expression

After the total surgical resection of the GBM tumor, retroviral vectors containing the HSV-TK gene are delivered via intracerebral injections into the remaining resection cavity [29]. The retroviral vectors will subsequently integrate the HSV-TK gene stably into the genome of dividing GBM cells to be expressed

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[13]. In HSV-TK expression, activator proteins are integral in promoting and maintaining transcription, the first of two steps during gene expression [30]. Activators, which are transcription factors, bind to specific sites known as promotors, specific DNA sequences typically upstream of the target gene [30, 31]. The promotor sequences of the HSV-TK gene are integrated alongside the HSV-TK gene during transduction in a therapeutic cassette [24]. Such promotors for HSV-TK act as binding sites to human cellular activators such as Sp1, CTF, and TFIID [32]. Another critical component in the expression and transcription of HSV-TK is enhancer DNA sequences. Enhancers are DNA sequences located up to a million base pairs away from the target gene, where other activator proteins bind [33]. DNA-bending proteins fold the DNA strand upon binding to bring the enhancer sequence near the promotor sequence [34]. The interacting activators on promotors and enhancer sequences can recruit RNA polymerase II (RNAP-II) to the promotor sequence, forming a transcription complex that initiates the transcription of the HSV-TK gene [35]. After initiation, RNAP-II escapes from the promotor sequence, moving down the HSV-TK gene to allow the elongation of the mRNA sequence [36]. A terminator sequence at the end of the gene exists, where RNAP-II breaks from the DNA strand, and the mRNA strand is cleaved [37].

Following transcription, the mRNA strand exits the nucleus to be translated, producing HSV-TK proteins. Ribosomes are tiny organelles of rRNA and proteins with two subunits [38]. The large upper subunit of the ribosome contains three sites for tRNA molecules, named the aminoacyl site (A site), the peptidyl site (P site), and the exit site (E site) [39, 40]. The ribosome initiation complex comprises the 40s small subunit and the methionine aminoacyl-tRNA (aa-tRNA), which has a complementary anticodon to the start codon. The initiation complex attaches to the 5’ end of the mRNA strand, moving towards the 3’ end until reaching the start codon. At the start codon, the upper subunit of the ribosome attaches to the initiation complex so that the methionine aa-tRNA sits in the P site. Notably, the methionine aa-tRNA is the only aa-tRNA molecule that initially enters the ribosome at the P site [39-41]. The ribosome then shifts to the next codon, and aa-tRNA molecules are recruited to the A site by the elongation factor eEF-1a until the aa-tRNA molecule with the complementary anticodon is recruited. When the complementary aa-tRNA is found, a GTP molecule on the ribosome is hydrolyzed to form GDP, and the eEF-1a elongation factor leaves [40]. Following complementary pairing, the peptidyl transferase center protein in the large subunit of the ribosome catalyzes two important reactions [42]. First, a peptide bond is formed between the amine (NH2) and carboxyl (COOH) groups of the amino acids on the aa-tRNA molecules in the A site and P site. Secondly, the peptidyl transferase center breaks the ester bond between the amino acid and the tRNA molecule in the P site [40, 42]. After these reactions, the ribosome shifts to the next codon. The tRNA molecule in the P site shifts to the E site and exits, while the aa-tRNA bonded to two amino acids in the A site moves to the P site [39]. A new aa-tRNA molecule with complementary anticodon is recruited to the A site by eEF-1a elongation factor [40]. This process is then repeated until a stop codon (UAA, UAG, and UGA) enters the A site of the ribosome. The release factor protein eRF1 recognizes and binds to the stop codon, and eRF3 catalyzes the hydrolysis of the polypeptide chain and tRNA in the P site, terminating translation [43]. The ribosome disassembles after translation termination to be recycled, and the peptide chain folds into the structure of the HSV-TK protein [40].

3.2. The Blood-Brain Barrier and Ganciclovir Metabolism

After HSV-TK transduction, GCV is administered intravenously, typically at a dose of 5mg/kg twice daily for two weeks [44]. Blood vessels in the brain are coated by a thin layer of various cells and proteins known as the blood-brain barrier (BBB). The BBB is vital in maintaining the brain's homeostasis, preventing toxin and pathogen entry, and controlling the movement of other molecules. The restrictive characteristics of the BBB are a significant obstacle in the treatment of brain tumors, as drug delivery to brain tissue is typically hindered [45]. However, nucleoside transport systems with broad specificity on the BBB enable the transport of nucleoside analogs such as GCV into brain tissue [46]. Upon entering cells without HSV-TK transduction, GCV remains a prodrug due to the lack of the HSV-TK protein involved in the first step of the metabolic pathway of GCV. GBM cells with HSV-TK

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transduction and expression catalyze the phosphorylation of GCV into GCV monophosphate [5]. Human cellular guanylate kinases will subsequently phosphorylate GCV monophosphate into GCV diphosphate [47, 48]. GCV diphosphate can be further phosphorylated by human cellular phosphoglycerate kinases (nucleoside diphosphate kinases) into GCV triphosphate (GCV-TP), a cytotoxic metabolite inhibiting DNA synthesis in GBM cells [48].

Deoxynucleotide triphosphate (dNTP) molecules, which act as precursors to DNA nucleotides, are vital in synthesizing DNA molecules. DNA polymerase molecules catalyze the transfer of a phosphate group on dNTP molecules, resulting in a pyrophosphate group attached to the deoxyribose sugar. The pyrophosphate group is hydrolyzed into two phosphate molecules, forming a DNA nucleotide [49]. One such dNTP molecule is deoxyguanosine triphosphate (dGTP), the triphosphate precursor to guanosine. The metabolite GCV-TP is an analog of dNTP, a competitive inhibitor of DNA polymerase, preventing the incorporation of guanosine into DNA [49, 50]. Notably, GCV-TK can inhibit both human cellular and viral DNA polymerase but more preferentially inhibits the latter [50]. GCV-TP molecules can also integrate into the replicating host cellular DNA [51]. This can lead to DNA double-strand breaks and the mitotic cell cycle arrest at the S, G2, and mitosis stages. The double-strand breaks due to GCV-TP incorporation provoke genomic instability, resulting in a decline of Bcl-2 expression, a family of primarily anti-apoptotic proteins [52, 53]. Bcl-2 proteins are important in regulating the permeability of the outer mitochondrial membrane. The downregulation of Bcl-2 allows the activation of the proteins BAX and BAK, activated by the BH3 protein, which was previously inhibited by Bcl-2 [53]. Upon activation, BAX and BAK proteins pierce through the outer mitochondrial membrane, leaving it porous. The pores formed in the outer mitochondrial membrane allow the release of cytochrome C (Cc), an essential pro-apoptotic protein, from the inner mitochondrial membrane [54]. Cc in the cytoplasm competes with 14-3-3 proteins in the binding of Apaf-1, a cytosolic protein that initiates the apoptosome assembly [55]. Seven bound Cc and Apaf-1 complexes form a heptameric ring structure, which recruits seven inactive pro-caspase-9 molecules. The pro-caspase-9 is activated, forming caspase-9 and completing apoptosome formation [56]. The active caspase-9 molecules activates other effector caspases molecules such as caspase-3, caspase-6, and caspase-7 [57, 58]. These activated effector caspases are protease molecules that cleave various critical cellular proteins, ultimately leading to the apoptosis and fragmentation of the target cell [56, 58].

3.3. The Bystander Effect

The bystander effect refers to a phenomenon observed in cancer cells in which the cytotoxic effects of gene therapy kill cells without gene transduction. The bystander function is a vital component in tumor regression as, in the best case, only 10% of tumor cells will express the HSV-TK enzyme [59]. Several studies have since investigated the bystander effect, and many have suggested the involvement of gap junction intercellular communication (GJIC) [7]. Gap junctions (GJ) are intermembrane channels that facilitate the flow of molecules such as HSV-TK between adjacent connecting cells. GJs are maintained by proteins named connexons, which are hexomers made of six connexins that span across the two surface membranes [60]. It has since been shown that cells exhibiting poor GJIC demonstrate the bystander effect after being co-transfected with connexin proteins to allow connexon formation [61]. GJIC in GBM cells with HSV-TK transduction results in the “kiss of death” function, in which the transportation of the cytotoxic GCV-TP kills cells without HSV-TK transduction [62].

Several other theories that may explain the bystander effect phenomenon have arisen. One proposes that the bystander effect results from the phagocytosis of apoptotic bodies of GBM cells containing the cytotoxic GCV-TP [61]. An investigation by Freeman et al. concluded that the apoptotic bodies of cancer cells with HSV-TK transduction were phagocytized by unmodified cells, resulting in the apoptosis of the unmodified cells [63]. However, this analysis remains inconclusive as GCV-TP could have potentially been transported to unmodified cells by GJIC before the phagocytosis of apoptotic bodies [61]. Another theory suggested that the bystander effect in cells could also depend on immune response. Agard et al. in 2001 formed tumors in two liver lobes in mice and injected HSV-TK into one of the lobes, followed by GCV administration. The tumor in both lobes showed regression, which could

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not have been explained by GJIC or the phagocytosis of apoptotic bodies [59]. Immunohistological analysis of the non-transducted liver lobe revealed the presence of CD8+ T-lymphocytes in the immune response induced by HSV-TK and GCV, essentially a "distant" bystander effect [64].

3.4. Limitations of HSV-TK Therapy in GBM

Significant limitations in the use of HSV-TK gene suicide therapy for GBM arise from the highly heterogeneous nature of GBM cells, which can have different tumor cell populations [65]. The presence of tumor heterogeneity in GBM significantly increases the difficulty of treatment. One such difficulty arises from the potential resistance of certain tumor cell populations to various cytotoxic drugs. For example, some of the populations of tumor cells in GBM may be resistant to cytotoxic compounds such as GCV-TP. Early progress and shrinking of the tumor mass may be observed as non-resistant tumor populations will be killed by GCV-TP. However, the remaining resistant cells will continue to proliferate and repopulate throughout the brain, resulting in tumor relapse of resistant tumor populations [66].

Another challenge in the treatment of GBM stems from the prominence of Tumor-associated macrophages (TAMs). Several studies have reported that 30-50% of cells in GBM are TAMs, while others suggest that the tumor microenvironment (TME) of GBM is dominated by TAMs [65, 67]. Increasing evidence suggests that TAMs in the TME directly promote tumor growth in various ways, which reduces the efficacy of cancer therapies [65]. TAMs directly produce several growth factors, like the epidermal growth factor, in the TME, enhancing tumor proliferation. In addition, TAMs can secrete cytokines that promote immunosuppression and activate transcription factors that increase the expression of anti-apoptotic proteins [68]. TAMs have also been proven to be essential in facilitating tumor angiogenesis as they actively secrete VEGF. VEGF promotes the migration of endothelial cells and the permeability of blood vessels to generate new vessels [65, 69].

4. Conclusion and Prospects

The treatment of GBM remains a challenging task despite decades of medical advances. Although the retroviral transduction of HSV-TK in the gene suicide therapy of GBM shows promising results, it is not without its limitations. Various studies and clinical trials have demonstrated that HSV-TK gene suicide therapy is effective in inducing tumor regression. GBM cells with HSV-TK transduction express the kinase enzyme, phosphorylating the prodrug GCV into a toxic metabolite GCV-TP. Notably, retroviral vectors can achieve this with minimal off-tumor cytotoxic effects due to their ability to target dividing cells specifically. Compared to other therapies, the reduced slide effects of HSV-TK therapy significantly increase the quality of life of GBM patients. HSV-TK therapy also offered improved therapeutic results and generally improved patient prognosis and life expectancy for GBM. However, the effectiveness of HSV-TK therapy is hindered severely by the heterogeneity of tumor populations and the aggressive nature of GBM due to the prevalence of TAMs in the TME.

One potential approach to improve the effectiveness of HSV-TK gene suicide therapy is to use it in tangent with therapies targeting TAMs. Given the integral role of TAMs in GBM progression, such a combined regimen could significantly halt GBM proliferation and growth. One such TAM-targeting drug is Zoledronate. Used mostly in treating bone metastasis, Zoledronate specifically attacks and depletes the pro-tumorigenic M2 TAMs, resulting in reduced immunosuppressive effects and slowing cancer proliferation [70]. Another approach to target TAMs is to prevent the recruiting of TAMs to the TME. For example, the Colony stimulating factor 1 receptor (CSF-1) is essential in recruiting and accumulating TAMs. Several Monoclonal antibodies and kinase inhibitors are being tested in clinical trials that inhibit the CSF-1 pathway, preventing TAM recruitment [70, 71].

Nonetheless, future research and trials should investigate and explore the possibility and efficacy of such combined regimens for GBM. Additionally, emphasis should be placed on research to better understand the TME heterogeneity of GBM. Better identification of GBM cell populations can allow treatments to be personalized and tailored to target populations resistant to common therapies, improving treatment efficacy and reducing GBM relapse.

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