Long-term treatment with valganciclovir improves lentiviral suicide gene therapy of glioblastoma

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Author contributions:

JAH and HM performed experiments and wrote the manuscript

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KR and BF provided lentiviral vectors and wrote the manuscript

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Abstract

Background: Suicide gene therapy for malignant gliomas has shown encouraging results in the latest clinical trials. However, prodrug application was most often restricted to short-term treatment (14 days), especially when replication-defective vectors were used. We previously showed that a substantial fraction of Herpes simplex virus thymidine kinase (HSV-TK) transduced tumor cells survive ganciclovir (GCV) treatment in an orthotopic glioblastoma (GBM) xenograft model. Here we analyzed whether these TK^{+} tumor cells are still sensitive to prodrug treatment and whether prolonged prodrug treatment can enhance treatment efficacy.

Methods: TK-GFP^{+} glioma cells were sorted from xenograft tumors recurring after suicide gene therapy, and their sensitivity to GCV was tested in vitro. GBM xenografts were treated with HSV-TK/GCV, HSV-TK/valganciclovir (valGCV) or HSV-TK/valGCV + erlotinib. Tumor growth was analyzed by MRI, and survival as well as morphological and molecular changes were assessed.

Results: TK-GFP^{+} tumor cells from recurrent xenograft tumors retained sensitivity to GCV in vitro. Importantly, a prolonged period (3 months) of prodrug administration with valganciclovir (valGCV) resulted in a significant survival advantage compared to short-term (3 weeks) application of GCV. Recurrent tumors from the treatment groups were more invasive and less angiogenic compared to primary tumors and showed significant upregulation of epidermal growth factor receptor (EGFR) expression. However, double treatment with the EGFR inhibitor erlotinib did not increase therapeutic efficacy.

Conclusions: Long-term treatment with valGCV should be considered as a replacement for short-term treatment with GCV in clinical trials of HSV-TK mediated suicide gene therapy.

Keywords:

Glioblastoma, Brain tumors, Suicide gene therapy, Lentiviral vectors, EGFR

Key Points:

- Prodrug administration scheme is a crucial factor in suicide gene therapy
- Prolonged prodrug administration results in improved survival

Importance of the study

Clinical trials for suicide gene therapy of glioblastoma with the HSV-TK/Ganciclovir (GCV) system used short-term prodrug treatment (14 days) as a standard regimen for suicide induction. In an orthotopic PDX model for glioblastoma we demonstrate that a substantial fraction of tumor cells expressing the suicide gene HSV-TK survive short-term treatment with the prodrug Ganciclovir. We verify that these tumor cells are not resistant to treatment, but retain sensitivity to GCV in vitro. We show that treatment with valganciclovir, an alternative prodrug allowing oral long-term administration significantly prolongs survival in a clinically relevant orthotopic PDX model. Thus, future clinical trials should consider long-term prodrug treatment to enhance the therapeutic effect of suicide gene therapy for glioblastoma.

Introduction

Glioblastoma (GBM) is the most aggressive primary brain tumor in humans. Standard therapy that involves maximal possible surgical resection followed by concomitant temozolomide chemotherapy and radiotherapy results in a median survival of 14.6 months [1]. Intrinsic heterogeneity [2] and local seclusion provided by the blood-brain-barrier (BBB), combined with the infiltrative nature make it difficult to develop targeted therapeutics for GBM.

Fairly efficient access of various prodrugs through BBB after systemic delivery has turned suicide gene therapy into a promising option for GBM treatment. Early clinical trials using replication-defective retroviral vectors encoding the suicide gene Herpes simplex virus thymidine kinase (HSV-TK) to transduce GBM cells followed by ganciclovir (GCV) application were disappointing, most likely due to low transduction efficiency [3,4,5,6]. One major drawback of retroviral vectors is the incapability of transducing quiescent cells. In contrast lentiviral vectors efficiently transduce quiescent cells which is mediated by the karyophilic nature of the lentiviral pre-integration complex [7]. Indeed, we observed significantly higher in vitro transduction rates of biopsy-derived and cancer-stem-like glioma cells compared to retroviral vectors [8]. We also showed that HSV-TK transduced normal brain cells are not killed by GCV prodrug treatment [9] and nonetheless can contribute to eliminating tumor cells through the bystander effect [10]. By using lentiviral suicide gene therapy in a biopsy-derived GBM xenograft model, we demonstrated complete remission of GBM on MRI with a significant survival benefit [8]. However, tumor recurrence was observed in all animals after termination of GCV administration. Importantly, we observed that a fraction of HSV-TK transduced tumor cells survived even after 4 weeks of GCV application [8]. At this point it was not known if HSV-TK in these tumor cells retained

functionality. In the present study, we show that the HSV-TK^{+} recurrent tumor cells harbor functional HSV-TK, which indicates that the duration of GCV application likely has not been long enough to efficiently target the slowly dividing or relatively-quiescent glioma cells. By using valganciclovir (valGCV) as a prodrug we confirmed our hypothesis showing significantly increased survival after long-term prodrug administration.

Materials and methods

Ethics statement

The Norwegian Data Inspectorate and the Regional Committee for Ethics in Research have approved this project (REK 013.09). The study was performed in accordance with the Helsinki Declaration.

Cells and spheroids

Biopsy material was obtained from resection surgery of a GBM patient (patient 3; P3) [11] in Haukeland University Hospital according to a similar protocol mentioned previously [12,13]. Resultant GBM spheroids were passaged in vivo by serial implantation in Rowett nude rats (RNU). Details of culture condition and generation of spheroids are described in the supplementary methods. Spheroids were implanted as described previously [8].

Animals

Healthy RNU rats were procured and provided with standard pellet diet and water *ad libitum*. Animals were treated in accordance with the Norwegian Animal Act. The local ethical committee approved the protocol.

Lentiviral vector construction, production and injection

Lentiviral vectors were constructed, prepared and injected intratumorally following protocols reported previously [14].

MRI

Details of brain MRI sequences have been reported previously [14].

Treatment

GCV (Cymeven; F. Hoffmann-La Roche) was given 50 mg/kg/day i.p. for 3 weeks and valGCV (Valcyte; F. Hoffmann-La Roche) was given 80 mg/kg/day P.O. for either 3 months or in cycles starting from 5 days after vector infusion. Erlotinib (Tarceva; F. Hoffmann-La Roche) was dissolved in acidified water (pH 4.8), mixed at an 1:1 ratio with 0.2% carboxymethylcellulose and was given P.O. 20 mg/kg/day for 5 days a week. This dosage resulted in side effects manifested by rash and weight reduction and led us to reduce the dose after 1 week to 5 mg/kg/day for 5 days a week which did not further result in any detectable side effects.

Immunohistochemistry and Immunofluorescence

Immunohistochemistry of paraffin sections was performed as described previously [8]. Immunofluorescence was performed as described in the supplementary methods. Dilution of primary antibodies is described in the supplementary methods. Images were acquired with Nikon E600 light microscope (Nikon Corporation) , NanoZoomer XR scanner (Hamamatsu Photonics) and Nikon widefield fluorescent microscope TE2000 (Nikon Corporation).

Digital analysis of images

Described in supplementary methods.

Statistics

Survival analysis was performed by Kaplan-Meier estimator with log rank test using graphpad prism software (GraphPad Software Inc). Other statistical analyses were performed by STATA software (StataCorp) or R (RStudio version 1.1.383) by t-test or ANOVA ($P<0.05$ was considered significant). Significant ANOVA results were followed by post-hoc pairwise comparisons with either Tukey's or Dunnett's adjustment for multiple comparisons. In the case of heteroscedasticity, dependent variables were log- or square root-transformed, depending on suitability.

Gene Expression Data Analysis

Whole-genome RNA sequencing was carried out at Macrogen Inc. (South Korea) on NovaSeq 6000 System (Illumina, Inc., San Diego). Details are described in supplementary methods.

Results

TK$^+$ glioma cells that survive short-term prodrug administration retain sensitivity towards GCV in vitro

Previously we have shown that a fraction of tumor cells expressing HSV-TK survive GCV prodrug treatment and are present in recurrent tumors [8]. These cells either may have become resistant to prodrug treatment or may be more quiescent than other tumor

cells in vivo. Here we investigated whether these remaining TK-transduced cells retain or lose sensitivity towards GCV. Intratumoral injection of lentiviral vectors encoding TK.007 [14], a recombinant HSV-TK with superior functionality [15,16], followed by GCV administration for 3 weeks resulted in tumor remission on MRI (Suppl. Fig. 1A). Recurrent tumors, which developed 7-8 weeks later, were dissociated and maintained in short-term culture. TK^+ cells were sorted from the cultures based on eGFP expression and immunostaining for human nestin confirmed that these cells were tumor cells of human origin (Fig. 1A). Treatment of the sorted TK^+ glioma cells with GCV in vitro showed that the cells retained sensitivity towards GCV (Fig. 1B). Upon further characterization of the cultured TK^+- and TK^- -glioma cells from the recurrent tumors, we observed no difference in terms of expression of glioma stem cell markers such as CD133, SOX2 and Nestin (Suppl. Fig. 1B). Similarly, there was no indication of a switch towards a mesenchymal or proneural subtype by using YKL40 and olig2 markers respectively (Suppl. Fig. 1B). In conclusion, TK^+ glioma cells do not become resistant to prodrug treatment over time.

Long-term valGCV administration improves the therapeutic effect of suicide gene therapy for experimental GBM

The sustained GCV sensitivity of the TK^+ cells indicated that the current prodrug scheme of 2-3 weeks of GCV treatment, which was also used in clinical trials for suicide gene therapy [6,17], is too short to eliminate all transduced tumor cells. To assess the effect of prolonged prodrug administration we introduced the drug valGCV, which is metabolized into GCV in the intestinal wall and liver [18]. valGCV is given orally and thus is suitable for long-term administration [19]. In contrast, GCV is given i.v. to patients by using catheters, which can cause infections upon long-term use [20]. We compared the

therapeutic benefit of long-term valGCV administration (3 months) with short-term (3 weeks) GCV treatment in the same orthotopic xenograft model. During treatment, the majority of animals from both treatment groups showed rapid reduction in tumor growth followed by tumor remission on MRI within 3-4 weeks (Fig. 2A). Kaplan-Meier survival analyses revealed that the TK/valGCV treatment group had a significant survival benefit compared to the TK/GCV group (Fig. 2B). Interestingly one animal from the valGCV treatment group was apparently cured and sacrificed healthy after more than 6 months of tumor remission (Suppl. Fig. 2A). All other animals in the valGCV treatment group developed recurrences distant from the primary site and often close to or in the brainstem (Suppl. Fig. 2B), which might explain why the difference in survival between the two treatment groups was not more extensive.

Continuous valGCV application eliminates the majority of TK$^+$ tumor cells

The majority of animals demonstrated tumor recurrence at various time periods after initial remission on MRI. While in the GCV treatment group 4 out of 4 animals showed local recurrences, all animals in the valGCV group showed recurrences that were distant from the primary site indicating that highly invasive cells escaped the treatment (Fig. 3A). Next, we analyzed whether TK-transduced cells had survived long-term treatment with valGCV. While TK-transduced cells were abundant in the recurrences of GCV treated animals, the distant recurrences of valGCV-treated animals were negative for the TK-transgene. However, scattered TK-positive cells were detected at the primary site of valGCV treated animals (Fig. 3B). When quantifying the number of transduced tumor cells on double immunofluorescence stained sections with antibodies against human nestin and GFP (Fig. 3C), we identified a significantly lower number of transduced residual glioma cells in the valGCV group compared to the GCV group

(Fig. 3D). Further, we observed that the residual TK^+ cells in the short-term GCV group are significantly less proliferative than the TK^- cells (Fig. 3E). We conclude that prolonged valGCV application facilitates the elimination of slow-proliferating glioma cells that are spared by current short-term treatment regimen.

Recurrent tumors are more invasive and less angiogenic compared to the primary tumors

Histological analyses revealed that the recurrent tumors from both treatment groups were more invasive and showed reduced vascularization/angiogenesis compared to the primary tumors from the control groups (Fig. 4A-F). Nestin immunohistochemistry revealed that GCV-treated tumors exhibited a diffuse growth pattern with extensive infiltration into the neighboring anatomical structures such as corpus callosum and even the contralateral hemisphere (Fig. 4A). Quantification of number of invasive cells outside the solid tumor mass confirmed that GCV-treated tumors were significantly more invasive compared to control tumors which both were found close to the primary injection site (Fig. 4B). All recurrent tumors of the valGCV group were found distant from the primary site which confirms that these tumors were initiated by highly invasive cells that escaped the treatment. When analyzing angiogenic/hypoxic features, primary tumors showed highly abundant necrotic areas and characteristic microvascular proliferations, which both were absent in local and distant recurrences (Fig. 4C). Immunostaining for vWF confirmed smaller vessels in the distant and local recurrent tumors compared to the primary tumors (Fig. 4D). Quantification of the vessel area fraction showed that the differences between primary and overall recurrent tumors (Fig. 4E), but not between the local and distant recurrences (Fig. 4F) were statistically significant. Apparently, there was an emergence of invasive glioma cells that escaped

prodrug treatment and established a secondary tumor mass mostly in brain regions distant from the primary site. Thus, we aimed at testing whether administration of valGCV in cycles (analogous to chemotherapy) might lower the threshold of surviving tumor cells for inducing an invasive escape program. We compared continuous valGCV treatment to cyclic valGCV using the experimental setup as described for Fig. 2. However the cyclic administration scheme neither showed any improved therapeutic effect nor resulted in reduced infiltrative nature of the recurrent tumors (Suppl. Fig. 3A and 3B).

Distant tumor recurrences show upregulation of EGFR and downregulation of pSTAT3

Due to the different growth pattern of distantly recurrent tumors in the valGCV group, we analyzed important molecular pathways involved in glioblastoma development by immunohistochemistry in order to identify proteins that might drive recurrent and invasive tumor growth. We performed immunostainings for (1) the tyrosine kinase receptors EGFR, PDGFR-A and c-MET, which often are amplified or co-amplified in GBM; (2) pMAPK and pSTAT3, which are activated downstream of tyrosine kinase signaling; (3) olig2, a protein abundantly expressed in glial tumors (Fig. 5 A,C,E and Suppl. Fig. 4A). c-MET, olig2 and pMAPK were equally expressed in all groups without obvious differences. However, there was a striking difference in EGFR expression: while the primary tumors were negative for EGFR, we observed EGFR expression in the recurrent tumors (Fig. 5A). Quantification of EGFR immunohistochemistry revealed that recurrent tumors expressed significantly higher EGFR in general and interestingly, distant recurrences further showed a significantly higher EGFR expression level compared to the local recurrences (Fig. 5B). To check if

the EGFR upregulation involved amplification events, we performed FISH for EGFR/CHR7 which only showed CHR7 gain, but no amplification in both treatment and control groups (Suppl. Fig. 4B). In addition to EGFR upregulation, we found differential expression of PDGFR-A, which exhibited uniform and abundant expression in the recurrent tumor tissue, while it was very heterogeneously expressed in primary tumors with strong expression in the vessel wall, but weaker in the tumor tissue (Fig. 5C). Quantification revealed that there was a significant difference in PDGFR-A expression between primary versus recurrent tumors, but not between local versus distant recurrences (Fig. 5D). pSTAT3, in contrast, was significantly downregulated in recurrent tumors compared with primary tumors (Fig. 5E,F). However, these differential expression patterns did not affect proliferative activity as shown by Ki-67 staining (Fig. 5G,H).

Recurrent tumors are resistant to Erlotinib treatment

Based on our observation that EGFR is upregulated in the recurrent tumors after gene therapy, we hypothesized that a combinatorial treatment regimen involving gene therapy plus anti-EGFR treatment would further improve the therapeutic benefit. To test this, we compared a combination therapy consisting of gene therapy plus erlotinib, a small-molecule EGFR inhibitor, with gene therapy alone. Resembling our previous animal experiments, tumor remission (on MRI) was observed within a 3-4 week time period in both treatment groups (data not shown) followed by tumor recurrences at later time points. Kaplan-Meier survival analysis showed that there was no survival difference between gene therapy-only and combination therapy (Fig. 6A). To analyze the effect of Erlotinib on EGFR phosphorylation we immunostained brain sections of the different groups with anti-pEGFR (1068) antibodies (Fig. 6B). We observed a trend

of reduced EGFR phosphorylation in the double treatment group compared to the gene therapy-only group (Fig. 6C). We also immunostained brain sections with an anti-EGFR antibody, which showed significant upregulation of EGFR expression in the combination treatment group compared to the gene therapy-only group (Fig. 6D, 6E). Thus, compensatory upregulation of EGFR in the tumor cells might impede the effect of anti-EGFR treatment.

Transcriptome profiling of recurrent tumors after TK+valGCV treatment

To identify global transcriptional changes associated with tumor recurrence after TK+valGCV treatment, we performed RNA sequencing. Interspecies variability of the sequences showed equal contamination by host tissue among all samples except one which was excluded (Suppl. Fig. 5A). Gene expression differential analysis revealed 262 genes upregulated and 139 genes downregulated (padj<0.05) when comparing the gene expression profile between the TK+valGCV and valGCV-only group (Suppl. Fig. 5B; Suppl. Fig. 6A,B; Suppl. table 1). Among the upregulated genes we detected EGFR (Suppl. Fig. 6B), confirming the previous results from immunohistochemistry in Figure 5. Biological processes/pathways associated with the transcriptional changes were identified by gene set enrichment analyses (GSEA). “Cellular response to hypoxia” and “vasculogenesis” were major processes associated with control tumors (Suppl. Fig. 6C, Suppl. tables 2 and 3) confirming our previous results showing increased angiogenesis in this group (Figure 4). Recurrent tumors from the TK+valGCV group exhibited upregulation of “Wnt signaling pathway” and “Cell adhesion molecules (CAMs)” among others (Suppl. Fig. 6D, Suppl. tables 2 and 3) indicating several invasive escape mechanism in addition to EGFR upregulation.

Discussion

In suicide gene therapy of brain tumors, 1-2 weeks of prodrug treatment is a standard regimen that by default, has been used in pre-clinical studies and clinical trials with replication-deficient vectors so far [3,6,17,21-24]. A similar prodrug administration scheme of maximum 2 weeks also dominates the ongoing clinical trials with replication-defective vectors (ClinicalTrials.gov Identifier: NCT03596086, NCT03603405, NCT00634231). This regimen had been tested extensively in animal models and had shown success there. However, tumors in animal models proliferate at a higher rate compared to the human situation, and in particular animal models based on serum-cultured monolayer cell lines exhibit a highly proliferative and non-invasive behavior [25]. The killing of tumor cells by GCV triphosphate (GCV-TP) is only mediated in proliferative cells as GCV-TP is incorporated as a nucleotide-analogon into the host DNA of dividing cells and terminates DNA synthesis. Thus, tumor cells are killed much faster in highly proliferating tumors compared to slow proliferating ones when using HSV-TK/GCV suicide gene therapy. For our study, we employed one of the most clinically relevant animal models for GBM [12] that closely recapitulates patient histology upon orthotopic implantation, retains major genetic changes of patient GBM and shows a similar proliferation rate [25]. Importantly, this PDX model shows profound heterogeneity at the single cell level and contains tumor cells from proneural, neural, classical and mesenchymal subtypes (unpublished data). Using this model system, we previously showed that lentiviral-vector mediated HSV-TK/GCV gene therapy is a highly promising therapeutic option for GBM [8]. However, a substantial fraction of TK^+ tumor cells were found to escape the standard GCV treatment. At that time, the

mechanism of survival of residual TK-transduced tumor cells remained open. Genomic deletion or transgene silencing were described as one potential mechanism of TK resistance after retroviral-vector mediated delivery[26], but expression of the transgene is still detectable in transduced cells that survived treatment in our model. Aberrant transcription [27] or splicing [28] can result in expression of non-functional TK. However, TK.007 involves codon corrections and optimizations, which remove the cryptic sites in question. Moreover, the extensive codon-optimization of TK.007 has removed many of the hot-spots for mutations described for wild-type TK [29], although the generation of potential new sites prone to to mutation could not be excluded.

However, we hypothesized that a fraction of the TK-expressing cells might become resistant through quiescence. In this work we showed that resistant TK^+ glioma cells indeed were slowly proliferating in vivo and retained sensitivity to ganciclovir in vitro. This data supported our assumption that the standard treatment of 2-3 weeks might be not long enough to kill all (or at least the vast majority of) transduced cells. In turn, it was suggestive to propose that a prolonged scheme of prodrug administration should result in better therapeutic outcome. However, systemic GCV treatment is associated with various drawbacks including cathether infections and thus is not a suitable option for long-term administration [18]. Instead, we decided to use valGCV as a prodrug, which is established as a preemptive treatment regimen in CMV disease and has been documented to be safe and well-tolerated by GBM patients after administration for more than 6 months [30]. valGCV is tailored as an oral formulation that readily gets metabolized to the active form (ganciclovir) in the intestinal wall and the liver [31]. Due to its similar efficacy compared to intravenous GCV, significantly higher bioavailability than oral GCV and safer mode of administration than intravenous GCV, valGCV has become a standard drug in clinical practice for CMV infection [18] .

In line with our hypothesis, we observed a significant survival benefit of the prolonged valGCV treatment regimen and significant reduction of TK^+ cells compared to GCV short-term treatment. However, except for one rat in the valGCV group, all other rats demonstrated tumor recurrence at various time points after initial tumor remission. Interestingly, the recurrence patterns between GCV and valGCV groups were spatially different. The distant recurrences of the valGCV-treated animals lacked tumor cells with TK.007-eGFP expression suggesting that highly invasive tumor cells that had not been transduced with TK escaped the treatment. Histological, transcriptomic and immunohistochemical analyses confirmed that recurrent tumors were more invasive and less angiogenic compared to the primary tumors while the primary tumors involved abundant necrotic areas and microvascular proliferation. Enhanced invasion of recurrent GBMs was also observed in other therapeutic settings such as bevacizumab treatment most likely induced by hypoxia [32-34]. In our setting, intermittent hypoxia could also play a role since lentiviral vectors can transduce endothelial cells [35], and killing these cells through HSV-TK/GCV could lead to less vascularization and hypoxia. Thus, similar mechanisms could be involved in inducing tumor invasion in different therapeutic settings.

Molecular characterization of the recurrent tumors by RNA sequencing and immunohistochemical analyses revealed upregulation of EGFR. The level of expression was also significantly higher in distant compared with local recurrences. EGFR was previously associated with tumor invasion in many different cancer types [36-39], and we previously showed that EGFR mediates invasion of tumor cells independent of angiogenesis [13,40]. Keunen et al. showed upregulation of EGF in bevacizumab treated GBM xenografts indicating that hypoxia and a lack of blood supply could be a potential mechanism for EGFR upregulation/activation [33]. Thus,

EGFR appeared to be an attractive target for a combinatorial treatment setting with suicide gene therapy to prevent tumor recurrence. Erlotinib is a prominent EGFR inhibitor that can cross the BBB [41-43] and downregulates EGFR activation (phosphorylation). There was a trend of reduced activation of EGFR upon erlotinib administration, but it was neither significant nor was it translated into therapeutic benefit (and /or reduced invasion of tumor cells). Inhibition of EGFR phosphorylation was reported to trigger various adaptive responses and thereby can resist therapy [44-46]. In line, single inhibition of EGFR by erlotinib was shown to be ineffective in a glioma PDX model unless combined with other inhibitors to target the corrsponding associated proteins that drive reactive compensatory mechanisms [45]. It is noteworthy in this context that gene enrichment analysis of RNA sequencing data revealed upregulation of additional genes/pathways in the TK+valGCV treated tumors including WNT signaling, which is known to be an important player of glioma invasion [47].

In conclusion, we show that the prodrug administration scheme is a key factor for suicide gene therapy of GBM. We report that long-term treatment with the prodrug valGCV improves HSV-TK suicide gene therapy for GBM by killing the tumor cells that still retain sensitivity towards the prodrug but are not eliminated by the standard short-term GCV treatment. Based on our results, prolonged and continuous administration of prodrug should be considered for suicide gene therapy studies and/or clinical trials for brain tumors in the future.

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Figure legends

Figure 1: TK$^+$ glioma cells that survive short-term prodrug administration retain sensitivity towards GCV in vitro. (A) Immunofluorescence staining with antibodies against human nestin (red) and GFP (green). Scale bar: 50 μm (B) Cell viability assay showing % of surviving cells after increasing concentrations of GCV; i.v.TK$^+$: The TK$^-$ cells present in the recurrent tumor were transduced with the same lentiviral vector in vitro.

Figure 2: Long-term administration of valGCV is more efficient compared to short-term GCV treatment in vivo. (A) Representative MRI (T2 RARE) images are shown. The solid tumor regions are marked with dashed lines. (B) Kaplan-Meier survival analysis: 3-month valGCV treatment has a significant survival benefit compared with the 3-week GCV treatment ($p=0.008$).

Figure 3: Continuous valGCV application eliminates the majority of TK$^+$ tumor cells. (A) H&E stainings of recurrences (left panel: recurrence at the primary site in GCV treatment group, right panel: recurrence distant from the primary site in valGCV treatment group) (B) Immunohistochemistry with antibodies for HSV-TK show transduced cells in the recurrent tumor mass. (C) Double immunofluorescent staining using antibodies against human nestin (green) and GFP (red) for the TK.007-eGFP fusion protein. Representative images show TK.007-eGFP$^+$ tumor cells that survived prodrug treatment. Scale bar: 50 μm. (D) Quantification of the remaining tumor cells expressing TK.007-eGFP: percentage of residual TK$^+$ tumor cells in 3-month long valGCV treated group is lower compared with the short-term GCV-treatment group

(p=0.042). (E) Proliferative capacity of the remaining TK^+ cells is significantly lower compared with $TK^—$ cells (p= 0.041) in the TK+GCV group.

Figure 4: Recurrent tumors after gene therapy are more invasive and less angiogenic compared with primary tumors. (A) Immunostaining with human nestin antibody showing extensive infiltration of glioma cells into corpus callosum in local and distant recurrent tumors, but not in the primary tumor. (B) Quantification of invasive tumor cells outside the solid tumor mass in control tumors and local recurrences (TK+GCV) (C) H&E stainings of primary and recurrent tumors showing necroses and microvascular proliferation only in the necrotic tumors. (D) vWF immunostaining reveals reduced angiogenesis in recurrent tumors compared to primary tumors. Scale bar 50 µm (E, F) Quantification of vessel area fraction among the groups. Overall recurrent tumors have significantly reduced vessel area compared to primary tumors (p=0.001).

Figure 5: EGFR is upregulated in recurrent tumors after suicide gene therapy. Immunohistochemical stainings with antibodies against EGFR (A), PDGFR-A (C), pSTAT3 (E) and Ki67 (G). Quantification showing percentage of $EGFR^+$ (B), PDGFR-A^+ (D) area fraction and $pSTAT3^+$ cells (F). Scale bar : 50 µm (H) Quantification of Ki-67^+ cells; mean±SD

Figure 6: Combination of suicide gene therapy with Erlotinib does not improve therapeutic efficacy. (A) Kaplan-Meier survival analysis does not show a significant difference between double treatment and single treatment arms. (B) Immunohistochemical stainings with antibodies against pEGFR 1068. Scale bar : 50

μm (C) Quantification showing percentage of pEGFR 1068^{+} area fraction (D,E) Immunohistochemical stainings with antibodies against EGFR and quantification of $EGFR^{+}$ area fraction.