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Detection of lentiviral suicide gene therapy in C6 rat glioma using hyperpolarised [1-¹³C]pyruvate

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ABSTRACT

Hyperpolarised [$1\text{-}^{13}\text{C}$]pyruvate MRI has shown promise in monitoring therapeutic efficacy in a number of cancers including glioma. In this study, we assessed the pyruvate response to the lentiviral suicide gene therapy of Herpes Simplex-1 thymidine kinase with the prodrug ganciclovir (HSV-TK/GCV) in C6 rat glioma and compared it with traditional MR therapy markers.

Female Wistar rats were inoculated with 10^6 C6 glioma cells. Treated animals received intratumoural lentiviral HSV-TK gene transfers on days 7 and 8 followed by two-week ganciclovir therapy starting on day 10. Animals were repeatedly imaged during therapy using volumetric MRI, diffusion and relaxation mapping, as well as metabolic [$1\text{-}^{13}\text{C}$]pyruvate MRS imaging. Survival (measured as time before animals reached a humane endpoint and were euthanised) was assessed up to day 30 post therapy.

HSV-TK/GCV gene therapy lengthened the median survival time from 12 to 25 days. This was accompanied by an apparent tumour growth arrest, but no changes in diffusion or relaxation parameters in treated animals. The metabolic response was more evident in the case-by-case analysis than in the group-level analysis. Treated animals also showed a $37\pm 15\%$ decrease ($p<0.05$, $n=5$) in lactate-to-pyruvate ratio between therapy weeks, whereas a $44\pm 18\%$ increase ($p<0.05$, $n=6$) was observed in control animals.

The hyperpolarised [$1\text{-}^{13}\text{C}$]pyruvate MRI can offer complementary metabolic information to traditional MR methods to give a more comprehensive picture of the slowly developing gene therapy response. This may benefit the detection of the successful therapy response in patients.

KEYWORDS: dissolution dynamic nuclear polarisation, metabolism, glioma, gene therapy, [$1\text{-}^{13}\text{C}$]pyruvate, herpes simplex thymidine kinase, ganciclovir

INTRODUCTION

Glioblastoma multiforme is one of the most aggressive incurable brain tumours, with most patients not surviving beyond 15 months after being diagnosed ¹. In addition to surgery combined with chemotherapy and radiotherapy, there is a continuing effort to develop novel treatments, such as targeted molecular therapies and metabolic modulation, with improved effectiveness ^{2,3}. For example, Herpes Simplex virus-1 thymidine kinase (HSV-TK) is the most comprehensively studied suicide gene therapy against malignant glioma ^{4,5}. HSV-TK catalyses the phosphorylation of nucleoside analog ganciclovir (GCV), converting it into a toxic metabolite which inhibits DNA replication and cell proliferation, thus inducing cell apoptosis ^{6,7,8}. The developing therapy regimen requires supporting diagnostic methods that can efficiently evaluate early treatment responses. Medical imaging is playing an increasingly important role in achieving this goal ⁹.

Traditionally therapy response is followed through changes in tumour volume. However, it is possible that changes in tumour metabolism, e.g. glycolytic rate, and other aspects of tumour biology can give an indication of the treatment response earlier than the tumour size ¹⁰. ¹⁸Fluoro-2-deoxyglucose positron emission tomography (FDG-PET) is the most widely used metabolic imaging technique for monitoring the treatment response in clinic. However, it may fail in brain, where the normal high tissue glucose uptake can mask the tumour glucose uptake ^{10,11}. Magnetic resonance spectroscopic (MRS) methods can be used to assess the elevated lactate (Lac) concentration in tumours ^{12,13}, which is closely linked with the glycolytic rate and, therefore, provides a diagnostic readout similar to FDG-PET ^{10, 14}. However, clinical lactate quantification using ¹H MRS can be compromised by relatively low signal-to-noise ratio and signal overlap from lipids present in the tumours.

Dissolution dynamic nuclear polarisation (dDNP) ¹⁵ hyperpolarised [^{1-¹³C}]pyruvate MRS imaging offers an alternative approach to non-invasive tumour lactate detection and treatment monitoring ^{16,17, 18}. Pyruvate is located at the metabolic branching point of glucose metabolism and, in tumours, is predominantly converted to lactate (“Warburg effect”) in a reaction catalysed by lactate dehydrogenase ^{19,20}. The appearance of hyperpolarised [^{1-¹³C}]lactate signal may therefore be a useful biomarker of altered metabolism in various metabolic diseases ¹⁰. It has been shown

that dDNP MRS can be used to detect elevated lactate in malignant rat glioma models, and that the lactate labelling is decreased following acute radiotherapy or chemotherapy^{17,21,22}. Recently, hyperpolarised [1-¹³C]pyruvate has been used for human malignant gliomas, demonstrating the clinical translational potential of the technique²³.

In the present study, we aimed to investigate the metabolic responses of malignant glioma to lentivirus mediated HSV-TK/GCV suicide gene therapy. Unlike the majority of dDNP studies so far, where a single dose of chemotherapy or radiation led to an acute tumour response, gene therapy response develops more slowly over several days. Therefore, our interest was to compare the metabolic response with traditional MR biomarkers during a two-week therapy period. In addition to volumetry, we also measured apparent diffusion coefficient and T₂ relaxation times, as they both have been used as microstructural biomarkers for preclinical gene therapy response²⁴, and may be useful also in conditions where tumour volume does not necessarily change. In particular, increased diffusion, usually associated with a decrease in cellularity, has been reported as a robust therapy marker in tumours. The data showed both volumetric and metabolic responses as a result of the therapy, but interestingly no effect on relaxation or diffusion properties of tissue water was detected. The results suggest that dDNP techniques can offer complementary information on tissue status during slowly developing gene therapy response.

METHODS

Vector construct and production

HSV-TK transgene expression was under constitutively active human phosphoglycerate kinase (hPGK) promoter²⁵. Lentiviral vector (LV) was produced in iCELLis 500 fixed-bed bioreactor (Pall Life Sciences, Hoegaarden, Belgium) by transfecting 293T cells (ATCC, Manassas, VA, USA) with four plasmid system required for third generation LV production by using PEIpro (Polyplus-transfection, Illkirch, France) mediated transfection as described in Leinonen et al. clarified by depth filtration (Merck Millipore, Espoo, Finland) and purified by tangential flow filtration using 100kDa cassette (Sartorius Stedim, Göttingen, Germany)²⁶. Final concentration was done by ultracentrifugation for 2h 10 min with 19 500 G and virus pellet was suspended

into phosphate buffered saline (PBS). Viral p24 capsid protein was measured using p24 ELISA (PerkinElmer Life Sciences, Turku, Finland).

In vitro cell viability assay and C6 cell culturing

C6 rat glioma cells (ECACC, Sigma-Aldrich, Helsinki, Finland) were seeded on 96-well plate, 2500 cells per well (day 0). Cells were let to adhere for 4 h and after that, the cells were transduced with LV vector expressing HSV-TK with a multiplicity of infection (MOI) 5, 10 or 20 or left non-transduced. The LV vector had a titre of 7.74×10^8 pg/ml (9.67×10^{12} virus particles/ml). On the following day (day 1) cells were treated with 1 µg/ml or 2 µg/ml ganciclovir hydrochloride (GCV, Cymevene® 500 mg, Roche, Espoo, Finland). Cell viability was measured at days 4 and 6 with a Cell Titer 96 AQueous One Solution Cell Proliferation Assay, MTS Assay (Promega/Labnet Oy, Helsinki, Finland) according to the manufacturer's instructions. Absorbance was detected at 490 nm with a Microplate reader using Ascent Software (Thermo Labsystems, Helsinki, Finland). Experiments were carried out with three or more replicates.

C6 glioma in Wistar rats

For in vivo experiments, C6 glioma cells were grown in a 10 cm petri-dish in 10 ml of high-glucose Dulbecco's modified eagle medium (DMEM, Sigma-Aldrich, UK) supplemented with 10 % fetal bovine serum and 1 % penicillin-streptomycin at 37 °C in the presence of 5 % CO₂. Upon reaching approximately 80 % confluence, the cells were washed twice with PBS and trypsinised with 1 ml of a 0.4 % trypsin solution. Cells were stained with a 0.4 % Trypan Blue solution at a dilution of 1:1 and counted using the Countess Automatic Cell Counter -machine (Invitrogen, Carlsbad, California, USA).

All animal experiments were approved by the Animal Health Welfare and Ethics Committee of the University of Eastern Finland. Female Wistar rats (n=46, 190 – 230 g, Envigo, Huntingdon, UK) were anaesthetised with intraperitoneal (i.p.) injection of ketamine 60 mg/kg (Ketalar vet 50 mg/ml, Pfizer, New York, New York, USA) and medetomidine hydrochloride 0.4 mg/kg (Domitor vet 1 mg/ml, Orion Pharma Animal Health, Espoo, Finland) and then secured into the stereotactic device (David Kopf Instruments, Tujunga, California, USA). Surgical implantation coordinates were 1 mm caudal from bregma and 2 mm to the right of sagittal suture. The 27G Hamilton syringe

(Hamilton, Bonaduz Ab, Switzerland) was lowered 2 mm below the top of bregma through a burr hole. C6 glioma cells were very slowly injected into the brain to avoid backflow. Furthermore, the syringe was left in place for a few minutes before lifting to avoid capillary effect. Post-operative pain medication (carprofen, Norocarp vet 50 mg/ml, Vet medic pharmaceuticals Oy, Parola, Finland) and anti-anesthesia (atipamezole hydrochloride, Antisedan vet 5 mg/ml, Orion Pharma Animal Health, Espoo, Finland) were administered subcutaneously according to general instructions.

Tumour growth was verified in a separate cohort of rats (n=15) before therapy experiments using volumetric MRI (see below). Dilutions were made to a final concentration of the C6 cell suspension of $1 \times 10^4 - 1.5 \times 10^6$ cells per 10 μ l of ice cold PBS. All animals implanted with 1×10^6 cells per 10 μ l or higher produced tumours within 8 days of implantation, whereas uptake was variable in lower concentrations (see supplementary table S1). The final concentration of 1×10^6 cells per 10 μ l of PBS was therefore selected for the subsequent therapy experiments (n=31).

***In vivo* gene transfer and therapy**

The timeline of gene transfer experiment is shown in figure 1. Animals were imaged with volumetric MRI (see the next section) on post-implantation (p.i.) day 6 to confirm the presence of tumours and to divide the animals into groups based on the tumour size. Tumours were divided between two groups so that each group contained as closely matched tumour sizes as possible (tumour volume ranges were 6-23 mm³ and 5-24 mm³ for therapy and control groups, respectively). Intratumoural gene transfers were conducted on both p.i. days 7 and 8 using a multiple-site vector injection technique. Animals in therapy group (n=15) were placed into the stereotactic device and 15 μ l volume of LV vector carrying HSV-TK was slowly injected into brain using a 27G Hamilton Syringe. Vector injections of 5 μ l per location were carried out at vertical depths of 1.5, 2.0 and 2.5 mm from the bregma. The needle was left in place for 5 min at each depth to allow proper dissipation of the vector solution. Starting on p.i. day 10, therapy rats received i.p. injections of GCV 25 mg/kg twice a day for 14 days. Control animals did not receive any treatment. All the animals were monitored daily and euthanised if any criteria for humane endpoint was reached (weight loss of over 20% from the original weight, or other significant cause of distress including paralysis or breathing problems).

¹H MRI and ¹³C dDNP

All MRI data were collected using a 9.4 T horizontal magnet interfaced to Agilent (Santa Clara, California, USA) imaging console. During the experiments, the animals were anaesthetised with isoflurane (5 % induction, 1-2 % upkeep) and placed inside a holder with a breathing monitor (60-80 breaths/min) and temperature control (37 °C) using warm water. For ¹³C experiments, a needle was placed into the tail vein of the animals for injection of hyperpolarised solution.

¹H data were collected using a volume coil transmitter/4-channel surface coil receiver pair (Rapid Biomedical, Rimpf, Germany). MR imaging was performed before GCV therapy was started (day -1 or 0, p.i. day 9 or 10), every 2-4 days during the therapy period, and then weekly until day 30 from the start of the therapy. Final MRI was performed prior euthanasia if humane endpoint was reached outside regular imaging intervals. ¹H imaging consisted of volumetric MRI (T₂-weighted multi-slice RARE, 32x32 mm² FOV with 0.125x0.125x1 mm³ voxel size, TR 3s, TE 72 ms at the center of k-space, 8 echoes with 12 ms echo spacing) and quantitative multi-slice parameter mapping (0.25x0.25x1.5 mm³ voxel size for all parameter maps, 9-12 slices) : T₁ (Look-Locker inversion-recovery FLASH, 12 inversion times between 5 and 5500 ms, 10 s delay between inversions, TR within FLASH 7.8 ms, TE 3.9 ms, 10 degrees flip angle), T₂ (multi spin-echo with 16 echoes collected between 8.1 and 129.8 ms, TR 2 s) and diffusion (diffusion prepared RARE, b-values 0 and 1000 s/mm² in three directions, TR 1.5 s, TE 22 ms, 8 echoes with 10.5 ms echo spacing, center of k-space on first echo, 4 averages) parameter mapping. All data were analysed in Matlab (Mathworks, Natick, Massachusetts, USA). Parameter maps were calculated using monoexponential fits and mean diffusion was estimated by averaging the data over three directions.

¹³C experiments were performed in a subset of therapy (n=7) and control (n=9) animals during the 14-day therapy period, with repeated imaging in the same animals on both weeks if possible. The first dDNP experiments were performed at day 5±1 and 4±1 days, in therapy and control groups respectively, the second experiment was performed 7 days later. For dDNP, 40 mg of neat (14 M) [1-¹³C]pyruvic acid (Sigma-Aldrich, Darmstadt, Germany) containing 22 mM AH11501 trityl radical (GE Healthcare, Chicago, Illinois, USA) was hyperpolarised for ~1.5 h at 6.7 T and 1.3 K

using HyperMag polariser (Technical University of Denmark, Copenhagen, Denmark)²⁷. Sample was dissolved using 13 ml of 200 mM TRIS buffer (pH 7.3) containing 0.1 g/L EDTA and neutralised with 434 µl of 1 M sodium hydroxide (NaOH) to yield a ~80 mM pyruvate solution with pH of ~7.5 and 20-30% polarisation levels, calculated from the ratio between the hyperpolarised signal and the corresponding thermal equilibrium signal observed in separate in vitro experiments. The polarisation levels are similar to those reported previously for pre-clinical experiments. ¹³C data were collected using a transmit/receive quadrature surface coil (Virtumed, Minneapolis, Minnesota, USA). A 6 mm axial slice was selected through the tumour based on the ¹H reference images collected using ¹H volume coil in a transmit/receive mode, in a few cases where the axial size of tumour was less than 6 mm in ¹H images, the ¹³C slice thickness was lowered to 4 mm. Chemical shift imaging (CSI, TR= 50 ms, TE=0.7 ms, FOV 32x32 mm with data matrix 16x16 spiral-out view order, spectral bandwidth 8000 Hz collected into 256 points) was initiated 30 s after intravenous injection was induced. Injection time was 10 s during which 5 ml/kg of hyperpolarised pyruvate (0.4 mmol/kg) was injected. All data analysis was performed in Matlab. Raw MR data were multiplied by a cosine function and zero-filled to 128 points in both spatial directions, line-broadened to 30 Hz and zero-filled to 4096 points in the spectral dimension before Fourier transformations, phase and baseline correction and peak integration. Data were analysed as lactate-to-pyruvate and lactate-to-bicarbonate ratios in the regions of interest in glioma and in normal brain. Lactate-to-pyruvate ratio probes the last step of the glycolytic pathway catalysed by lactate dehydrogenase enzyme and has been shown to be a useful therapy marker¹⁰. A decrease in lactate-to-bicarbonate ratio has recently been suggested as an alternative marker for therapy response in C6 gliomas²⁸. The ratio probes the relative balance between glycolysis and oxidative phosphorylation and furthermore uses signals that are from predominantly intracellular molecules, thereby making it less sensitive to perfusion effects.

Blood sampling

Blood samples for full blood count analysis and the assessment of liver and kidney functions were collected after completing the treatment period in order to detect the possible toxicity of the therapy. Samples were analysed at Movet veterinary analysis laboratory (Kuopio, Finland).

Histological analysis

Brains were collected for histology after euthanasia either due to humane endpoint being reached or at the end of the MR follow-up. Paraffin embedded brain sections of 4 μm were stained with haematoxylin and eosin for general overview and morphology of the tumours.

Statistical analysis

Statistical analyses were performed using GraphPad Prism Version 5.03 (GraphPad Software Inc., San Diego, California, USA) and Microsoft Excel 2016 (Microsoft, Seattle, Washington, USA). The results are reported as mean $\pm$ standard error of mean (SEM). Statistical significance between group means was estimated using Student's t-test assuming equal group variances, $p < 0.05$ was deemed significant. For repeat measures in the same animals, a paired test was used. Statistical significance of difference in survival times was estimated using Log-rank and Gehan-Breslow-Wilcoxon test for survival.

RESULTS

Cell viabilities of transduced C6 glioma cells after GCV treatment *in vitro*

The functionality of LV vector carrying HSV-TK in combination with GCV was tested in cell viability assay by transducing C6 cells with an increasing number of vector and analysing viability at days 4 and 6. LV mediated HSV-TK/GCV gene therapy significantly decrease the viability of C6 glioma cells over a different range of MOIs (5, 10, 20) up to 6 days after transduction (Fig 2). There was less than 30 % of living cells left after suicide gene therapy at day 4, while the viability of treated cells at day 6 was less than 10 %. Either gene transfer or GCV treatment alone had only minor effect of viability.

In vivo gene therapy studies

Histopathological overview of developed rat C6 gliomas revealed malignant tumours which represent multiple characteristics of human malignant glioma (Fig. 3a) ²⁹. Numerous mitoses occurred in tumour areas. Mitotic activity is one of the hallmarks of malignant tumour and could be used as a prognostic factor in diagnosis. Histology

showed fuzzy borders of the tumours and they were identified as World Health Organisation (WHO) grades III or IV according to their appearances.

Gene therapy resulted in an improved survival of animals with the median survival times of 12 and 25 days for control and therapy groups, respectively ($p < 0.05$ Gehan-Breslow-Wilcoxon test) (Fig. 3b). The animals lost during the 30-day follow-up period either were sacrificed due to humane endpoint, in most cases weight loss, being reached ($n=10$ control group and $n=9$ therapy group) or died during MRI due to a sudden loss of breathing ($n=1$ in each group). 5 control animals and 5 treated animals were alive at the end of the follow-up period. Virus transfection led to an immediate weight-loss in the therapy group followed by relatively stable weight development, whereas a slower weight decrease was seen in the control group, most likely due to increasing tumour burden (Figure 3c). After D14, a similar weight increase was observed in the surviving animals in each group. Prior to therapy (p.i. days 9-10), mean tumour volume was $58 \pm 10 \text{ mm}^3$ ($n=31$) with no difference in tumour volumes between the study groups. An apparent tumour growth arrest was seen in the therapy group (Fig. 3d), leading to a tumour volume difference between the animal groups from therapy day 3 onwards ($p < 0.05$). At later time points, some signs of tumour shrinkage was observed in surviving animals. Water diffusion and relaxation data showed no significant changes in the therapy group, whereas an increase of apparent diffusion coefficient and T_2 relaxation time was observed in the control tumours at the later time points (Table 1).

High lactate signal was observed from tumours (Fig. 4a and 4b). In dDNP experiments, mean lactate-to-pyruvate ratios during the first week of therapy were 0.83 ± 0.17 ($n=6$) and 0.79 ± 0.13 ($n=8$) for therapy and control groups, respectively, with no difference between the study groups (Fig. 4c). For comparison, in the contralateral brain the lactate-to-pyruvate ratio was 0.44 ± 0.05 ($n=14$). On the second week, the mean tumour lactate-to-pyruvate ratios were 0.67 ± 0.27 ($n=6$) and 1.16 ± 0.25 ($n=7$) for therapy and control groups, respectively. Four out of five treated animals imaged at both weeks showed a decreasing trend in lactate-to-pyruvate ratio (Fig. 4d) from the first to the second week (mean change in lactate-to-pyruvate ratio over all 5 animals, -0.27 ± 0.17 [$-37\% \pm 15\%$], $p < 0.05$), whereas an opposite effect was observed in five out of six control animals (mean change over all 6 animals, $+0.40 \pm 0.19$ [$+44\% \pm 18\%$], $p < 0.05$),

leading to a significant difference between animals in therapy and control group ($p < 0.01$). The treated animal showing the increasing lactate-to-pyruvate ratio did not show treatment response in volumetry either, suggesting unsuccessful therapy. During the first week of therapy, the mean lactate-to-bicarbonate ratio was 27 ± 6 ($n=14$) with no difference between the groups. During the second week, no change in ratio was seen in the therapy group ($n=4$, one animal showed too low bicarbonate signal to calculate ratio) but an increasing ratio was observed in the control group (change in ratio $+38 \pm 21$ [$+120 \pm 40\%$], $p < 0.05$, $n=6$).

Blood values

There were no significant differences either in full blood samples or liver and kidney functions between the study groups (Fig. S1).

DISCUSSION

In the current study, LV mediated HSV-TK/GCV suicide gene therapy led to both anatomic and metabolic changes in C6 tumours, as well as improved survival. We observed a metabolic response to the gene therapy using hyperpolarised pyruvate, although this did not appear to precede a slowdown of tumour growth, as detected through traditional volumetric analysis. However, gene therapy resulted in no alterations of tumour water relaxation or diffusion properties, which are often used as biomarkers for preclinical tumour therapy response²⁴. The results indicate that dDNP techniques can offer complementary information on slowly occurring gene therapy response.

Therapy resulted in a decreased tumour lactate signal following injection of hyperpolarised pyruvate. In contrast, increased lactate signal was generally seen in control tumours. The current results are consistent with other studies, including previous works with C6 model^{9,16,17,21,22}, demonstrating that lowered lactate labeling is a relatively universal marker of a cancer therapy. Nonetheless, there was a relatively large within-group variation between animals (Fig. 3c). It is possible, that this reflects biological variation between tumours with a potential additional confounding effect from perfusion since the imaging was performed in a single fixed time point (30s). The latter might be reduced by dynamic imaging following [$1\text{-}^{13}\text{C}$]pyruvate injection, which would allow a better estimation of apparent pyruvate-lactate exchanges rates using

more extensive signal modelling ^{9,16}. However, the dynamic approach is technically more demanding, especially at high magnetic fields, and is therefore more prone to errors related to bandwidth and signal-to-noise issues, especially for bicarbonate signal. Previous studies suggest that imaging at 30 s is a reasonable time point for detecting therapy response at C6 tumours ¹⁷. The therapy effect was more clearly visible when the changes within individual animals were compared (Fig. 3d), a situation more closely reflecting clinical settings. Similar therapy responses have been recently observed in prostate cancer patients ³⁰. The lactate-to-pyruvate and lactate-to-bicarbonate ratios observed in our study matched lactate-to-total-carbon ratios reported previously in C6 model (e.g. lactate-to-pyruvate ratio of 0.79 corresponds approximately to 0.43 lactate-to-total-carbon ratio) ³¹. However, in our experiments, the lactate-to-pyruvate ratio gave more robust therapy readout than the lactate-to-bicarbonate ratio. This may reflect metabolic differences between acute dichloroacetate ²⁸ or anti-VEGF therapy ²⁸ and more slowly developing gene therapy response. The therapy led to a growth arrest, which could be linked to an overall slowing down of tumour metabolism, leading to lowered lactate-to-pyruvate ratio, but not necessarily to an alteration in the relative rates of glycolysis and oxidative phosphorylation. On the other hand, the division with the relatively small bicarbonate signal (approximately 1% of total carbon signal) makes this ratio more prone to large variation in values, which could mask small metabolic effects. This could be seen especially in control tumours, where bicarbonate signal almost disappeared in the largest tumours.

Due to time and technical limitations, not all the animals could undergo simultaneous dDNP and ¹H MRI at all time points. Nevertheless, the data suggest that in our conditions the metabolic response did not precede the slowdown in tumour growth. This may partially reflect the inherent variation in metabolic ratios observed between the animals, and the sensitivity of the metabolic response might therefore be improved by imaging each animal before the start of the treatment to allow them to serve as their own controls. In the current study, this was not done to avoid excessive number of injections during the course of the study. The exact mechanism leading to the loss of lactate labelling cannot be determined from the current data, but likely involves a combination of changes in pyruvate delivery, glycolytic activity and, as a consequence, intratumoural lactate concentration. The increased lactate labelling observed in control

tumour has been previously associated with worsening malignancy of tumours ³¹. Traditional MR analysis demonstrated that gene therapy led to a growth arrest during the therapy, rather than to a widespread cell death and tumour shrinkage. Minimal changes in relaxometry and diffusion properties following therapy suggest that microstructural changes (e.g. cellularity) ²⁴ are not likely to dominate the dDNP therapy response. A slight increase in diffusion coefficient and relaxation times were observed in control tumours at later time points. This may be due to increased necrosis, present in histology, as a result of increased tumour size or a result of the death due to inherent immune response.

There are some potential limitations in the current study. In our experiments, treated animals were compared with control animals that did not receive any treatment. Both HSV-TK and GCV alone could trigger cell death, although we saw very little effect in vitro (Fig. 1). However, we cannot rule out an in vivo effect from the individual components of gene therapy. In most animals, tumour growth tended to restart after the therapy period leading to an increased median survival time but not cure. This is consistent with previous gene therapy studies ^{32,33} and may reflect factors such as limited spread of virus inside the solid tumours. A fraction of control animals also survived the full 30-day surveillance period and there were also signs of tumour shrinkage at later time points. This likely reflects the inherent immune response that has been reported for C6 model due to its background in outbred Wistar rat ^{34,35}. In this study, C6 glioma cell line was selected because it has been extensively used in dDNP therapeutic studies. Literature on C6 model varies in regards to the appearance of the immune response, and to our knowledge, previous dDNP studies on the same model ^{9,16,17,21,22} have not mentioned this phenomenon. Preferably, an inbred model such as a syngeneic BT4C glioma in BDIX rat could be used in gene therapy studies ^{36,37}. On the other hand, previous studies have suggested hyperpolarised pyruvate as a potential biomarker for immunogenic events ³⁸. The inherent immunogenic responses against C6 cells could, therefore, offer an interesting model to look into the metabolism related to immune responses.

The results indicate that dDNP techniques can offer complementary information on the tissue metabolic status of the slowly occurring gene therapy response. Although in the current model the metabolic information did not improve the response readout

beyond to that observed using volumetry, it might, therefore, be useful in situations where gross morphometric changes are not usually observed.

Conflict of interest

MIK shares intellectual property related to dDNP techniques with GE Healthcare.

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REFERENCES

1. Kazda T, Dziacky A, Burkon P, Pospisil P, Slavik M, Rehak Z, Jancalek R, Slampa P, Slaby O, Lakomy R. Radiotherapy of glioblastoma 15 years after the landmark Stupp's trial: More controversies than standards? *Radiol Oncol*. 2018;52(2):121-128.
2. Urbanska K, Sokolowska J, Szmids M, Sysa P. Glioblastoma multiforme - an overview. *Contemp Oncol (Pozn)*. 2014;18(5):307-312.
3. Birk HS, Han SJ, Butowski NA. Treatment options for recurrent high-grade gliomas. *CNS Oncol*. 2017;6(1):61-70.
4. Immonen A, Vapalahti M, Tyynela K, Hurskainen H, Sandmair A, Vanninen R, Langford G, Murray N, Ylä-Herttuala S. AdvHSV-tk gene therapy with intravenous ganciclovir improves survival in human malignant glioma: A randomised, controlled study. *Mol Ther*. 2004;10(5):967-972.
5. Westphal M, Ylä-Herttuala S, Martin J, Martin J, Warnke P, Menei P, Eckland D, Kinley J, Kay R, Ram Z; ASPECT Study Group. Adenovirus-mediated gene therapy with sitimagene ceradenovec followed by intravenous ganciclovir for patients with operable high-grade glioma (ASPECT): A randomised, open-label, phase 3 trial. *Lancet Oncol*. 2013;14(9):823-833.

6. Moolten FL. Tumor chemosensitivity conferred by inserted herpes thymidine kinase genes: Paradigm for a prospective cancer control strategy. *Cancer Res.* 1986;46(10):5276-5281.
7. Fischer U, Steffens S, Frank S, Rainov NG, Schulze-Osthoff K, Kramm CM. Mechanisms of thymidine kinase/ganciclovir and cytosine deaminase/ 5-fluorocytosine suicide gene therapy-induced cell death in glioma cells. *Oncogene.* 2004;24:1231.
8. Chen Y, Huang H, Yao C, Fengbo S, Wenming G, Shijun Y, Zhaohui N. Antitumor activity of combined endostatin and thymidine kinase gene therapy in C6 glioma models. *Cancer Med.* 2016;5(9):2477-2486.
9. Park JM, Josan S, Jang T, Merchant M, Yen YF, Hurd RE, Recht L, Spielman DM, Mayer D. Metabolite kinetics in C6 rat glioma model using magnetic resonance spectroscopic imaging of hyperpolarized [1-(13)C]pyruvate. *Magn Reson Med.* 2012;68(6):1886-1893.
10. Brindle KM, Bohndiek SE, Gallagher FA, Kettunen MI. Tumor imaging using hyperpolarized 13C magnetic resonance spectroscopy. *Magn Reson Med.* 2011;66(2):505-519.
11. Gambhir SS. Molecular imaging of cancer with positron emission tomography. *Nature Reviews Cancer.* 2002;2:683.
12. Sijens PE, Levendag PC, Vecht CJ, Dijk Pv, Oudkerk M. 1H MR spectroscopy detection of lipids and lactate in metastatic brain tumors. *NMR Biomed.* 1996;9(2):65-71.
13. Sibtain NA, Howe FA, Saunders DE. The clinical value of proton magnetic resonance spectroscopy in adult brain tumours. *Clinical Radiology.* 2007;62(2):109-119.
14. Terpstra M, Gruetter R, High WB, Mescher M, DelaBarre L, Merkle H, Garwood M. Lactate turnover in rat glioma measured by in vivo nuclear magnetic resonance spectroscopy. *Cancer Research.* 1998;58(22):5083-5088.
15. Ardenkjaer-Larsen JH, Fridlund B, Gram A, Hansson L, Lerche MH, Servin R, Thaning M, Golman K. Increase in signal-to-noise ratio of < 10,000 times in liquid-state NMR. *Proceedings of the National Academy of Sciences of the United States of America.* 2003;100(18):10158-10163.

16. Day SE, Kettunen MI, Gallagher FA, Hu DE, Lerche M, Wolber J, Golman K, Ardenkjaer-Larsen JH, Brindle KM. Detecting tumor response to treatment using hyperpolarized ^{13}C magnetic resonance imaging and spectroscopy. *Nature medicine*. 2007;13(11):1382-1387.
17. Day SE, Kettunen MI, Cherkuri MK, Mitchell JB, Lizak MJ, Morris HD, Matsumoto S, Koretsky AP, Brindle KM. Detecting response of rat C6 glioma tumors to radiotherapy using hyperpolarized 1- ^{13}C]pyruvate and ^{13}C magnetic resonance spectroscopic imaging. *Magnetic Resonance in Medicine*. 2011;65(2):557-563.
18. Park JM, Josan S, Jang T, Merchant M, Watkins R, Hurd RE, Recht LD, Mayer D, Spielman DM. Volumetric spiral chemical shift imaging of hyperpolarized [2-(^{13}C)c]pyruvate in a rat c6 glioma model. *Magn Reson Med*. 2016;75(3):973-984.
19. Warburg O. On the origin of cancer cells. *Science*. 1956;123(3191):309.
20. Vander Heiden MG, Cantley LC, Thompson CB. Understanding the warburg effect: The metabolic requirements of cell proliferation. *Science*. 2009;324(5930):1029.
21. Park I, Larson PEZ, Zierhut ML, Hu S, Bok R, Ozawa T, Kurhanewicz J, Vigneron DB, Vandenberg SR, James CD, Nelson SJ. Hyperpolarized ^{13}C magnetic resonance metabolic imaging: Application to brain tumors. *Neuro-oncology*. 2010;12(2):133-144.
22. Park I, Bok R, Ozawa T, Phillips JJ, James CD, Vigneron DB, Ronen SM, Nelson SJ. Detection of early response to temozolomide treatment in brain tumors using hyperpolarized ^{13}C MR metabolic imaging. *J Magn Reson Imaging*. 2011;33(6):1284-1290.
23. Gordon JW, Chen HY, Autry A, Park I, Van Criekinge M, Mammoli D, Milshteyn E, Bok R, Xu D, Li Y, Aggarwal R, Chang S, Slater JB, Ferrone M, Nelson S, Kurhanewicz J, Larson PEZ, Vigneron DB. Translation of carbon-13 EPI for hyperpolarized MR molecular imaging of prostate and brain cancer patients. *Magn Reson Med*. 2019;81(4):2702-2709.
24. Kettunen MI, Gröhn OH. Tumour gene therapy monitoring using magnetic resonance imaging and spectroscopy. *Curr Gene Ther*. 2005;5(6):685-696.
25. Leinonen HM, Ruotsalainen AK, Maatta AM, Laitinen HM, Kuosmanen SM, Kansanen E, Pikkarainen JT, Lappalainen JP, Samaranayake H, Lesch HP, Kaikkonen MU, Ylä-Herttuala

S, Levonen AL. Oxidative stress-regulated lentiviral TK/GCV gene therapy for lung cancer treatment. *Cancer Res.* 2012;72(23):6227-6235.

26. Leinonen HM, Lipponen EM, Valkama AJ, Hynynen H, Oruetxebarria I, Turkki V, Olsson V, Kurkipuro J, Samaranayake H, Määttä AM, Parker NR, Ylä-Herttuala S, Lesch HP. Preclinical proof-of-concept, analytical development, and commercial scale production of lentiviral vector in adherent cells. *Molecular Therapy - Methods & Clinical Development.* 2019;15:63-71.

27. Ardenkjaer-Larsen JH, Bowen S, Petersen JR, Rybalko O, Vinding MS, Ullisch M, Nielsen NC. Cryogen-free dissolution dynamic nuclear polarization polarizer operating at 3.35 T, 6.70 T, and 10.1 T. *Magn Reson Med.* 2019;81(3):2184-2194.

28. Park JM, Spielman DM, Josan S, Jang T, Merchant M, Hurd RE, Mayer D, Recht LD. Hyperpolarized ^{13}C -lactate to ^{13}C -bicarbonate ratio as a biomarker for monitoring the acute response of anti-vascular endothelial growth factor (anti-VEGF) treatment. *NMR Biomed.* 2016;29(5):650-659.

29. Louis DN, Perry A, Reifenberger G, von Deimling A, Figarella-Branger D, Cavenee WK, Ohgaki H, Wiestler OD, Kleihues P, Ellison DW. The 2016 world health organization classification of tumors of the central nervous system: A summary. *Acta Neuropathol.* 2016;131(6):803-820.

30. Aggarwal R, Vigneron DB, Kurhanewicz J. Hyperpolarized 1-[(^{13}C)]-pyruvate magnetic resonance imaging detects an early metabolic response to androgen ablation therapy in prostate cancer. *Eur Urol.* 2017;72(6):1028-1029.

31. Albers MJ, Bok R, Chen AP, Cunningham CH, Zierhut ML, Zhang VY, Kohler SJ, Tropp J, Hurd RE, Yen YF, Nelson SJ, Vigneron DB, Kurhanewicz J. Hyperpolarized ^{13}C lactate, pyruvate, and alanine: Noninvasive biomarkers for prostate cancer detection and grading. *Cancer Res.* 2008;68(20):8607.

32. Sandmair AM, Loimas S, Puranen P, Immonen A, Kossila M, Puranen M, Hurskainen H, Tyynelä K, Turunen M, Vanninen R, Lehtolainen P, Paljärvi L, Johansson R, Vapalahti M, Ylä-Herttuala S. Thymidine kinase gene therapy for human malignant glioma, using replication-deficient retroviruses or adenoviruses. *Hum Gene Ther.* 2000;11(16):2197-2205.

33. Miletic H, Fischer YH, Girolou T, Rueger MA, Winkeler A, Li H, Himmelreich U, Stenzel W, Jacobs AH, von Laer D. Normal brain cells contribute to the bystander effect in suicide gene therapy of malignant glioma. *Clin Cancer Res*. 2007;13(22 Pt 1):6761-6768.
34. Barth RF, Kaur B. Rat brain tumor models in experimental neuro-oncology: The C6, 9L, T9, RG2, F98, BT4C, RT-2 and CNS-1 gliomas. *J Neurooncol*. 2009;94(3):299-312.
35. Biasibetti E, Valazza A, Capucchio MT, Annovazzi L, Battaglia L, Chirio D, Gallarate M, Mellai M, Muntoni E, Peira E, Riganti C, Schiffer D, Panciani P, Lanotte M. Comparison of allogeneic and syngeneic rat glioma models by using MRI and histopathologic evaluation. *Comp Med*. 2017;67(2):147-156.
36. Sandmair AM, Turunen M, Tyynela K, Loimas S, Vainio P, Vanninen R, Vapalahti M, Bjerkvig R, Jänne J, Ylä-Herttuala S. Herpes simplex virus thymidine kinase gene therapy in experimental rat BT4C glioma model: Effect of the percentage of thymidine kinase-positive glioma cells on treatment effect, survival time, and tissue reactions. *Cancer Gene Ther*. 2000;7(3):413-421.
37. Samaranayake H, Olsson V, Kurkipuro J, Määttä A-M, Sted H, Kärkkäinen H-R, Kaikkonen MU, Taavitsainen M, Vuorio T, Parker N, Ylä-Herttuala S. Up-regulation of mismatch repair pathway by suicide gene therapy: Implications on the use of temozolomide treatment in malignant glioma. *Journal of Cancer Science & Therapy*. 2018;10:214-222.
38. MacKenzie JD, Yen Y, Mayer D, Tropp JS, Hurd RE, Spielman DM. Detection of inflammatory arthritis by using hyperpolarized ¹³C-pyruvate with MR imaging and spectroscopy. *Radiology*. 2011;259(2):414-420.

Figure legends

Figure 1. Timeline of gene therapy studies, day 0 is the start of GCV therapy. Animals were divided into control and therapy groups based on tumour size, as assessed with MRI, at D-4. Therapy animals received gene transfers on both days D-3 and D-2, and GCV therapy from D0 to D14. Follow-up ended at D30.

Figure 2. Cell viability (%) of C6 rat glioma cells at days 4 and 6 of LV mediated HSV-TK/GCV suicide gene therapy. Cells were either non-transduced (NT) or transduced with different concentrations of the treatment vector (HSV-TK), followed by GCV treatment. Cell viability was measured by the MTS assay. NT, non-transduced; MOI, multiplicity of infection; HSV-TK, Herpes-Simplex virus-1 thymidine kinase; GCV, ganciclovir. Values shown as mean $\pm$ SEM (n=3).

Figure 3. Representative MRI and histological slide of haematoxylin and eosin (H&E) stained C6 glioma (a), as well as survival (b), body weight development from implantation (c) and volumetric (d) data in two study groups. Animals were sacrificed either when the humane endpoint was reached or at day 30. In panels b-d, the end of GCV therapy is marked with a vertical line. Panel c shows weight development in the surviving animals (symbols) and all the studied animals (dotted lines, therapy n=15, control n=16. The weights of euthanised animals are counted as -20% based on the definition of the humane endpoint). Time points for dDNP experiments (see Fig. 4) are also shown in panel d. Histology showed increased mitosis (see inset) as well as fuzzy borders indicating malignant appearances. HSV-TK/GCV therapy led to improved survival (b), some initial weight loss (c), and a tumour growth slowdown (d). Values in panels c and d are given as mean $\pm$ SEM (the number of animals in each time point was decreasing according to the survival data shown in panel b), * p<0.05, ** p<0.01 (Student's t-test).

Figure 4. Representative CSI dataset showing pyruvate and lactate distributions, tumour region is indicated with an arrow (a), and a spectrum from tumour region (b). Box-and-whisker plots (whiskers represent min-max values) for lactate-to-pyruvate

ratio measured in individual tumours during GCV therapy (days 0-14) (c) and the slope of ratio change between repeated measurements in the same animals (d). The first dDNP experiments were performed at day 5 ± 1 and 4 ± 1 days (mean $\pm$ SEM), in therapy and control groups respectively, the second experiment was performed 7 days later. Four out of five treated animals showed a negative slope, whereas five out of six control animals had a positive slope, leading to a statistically significant difference ($p < 0.05$) between the groups.

Table 1. Tumour tissue water relaxation times and apparent diffusion coefficients during the therapy

	T₁ (s)		T₂ (ms)		D_{AV} (*10⁻³ mm²/s)	
	Therapy	Control	Therapy	Control	Therapy	Control
DAY 0	2.06 ± 0.12 (n=7)	2.16 ± 0.04 (n=11)	55 ± 1 (n=12)	59 ± 2 (n=15)	0.78 ± 0.03 (n=10)	0.77 ± 0.03 (n=15)
DAY 3-4	2.14 ± 0.10 (n=9)	2.12 ± 0.07 (n=12)	58 ± 1 (n=15)	62 ± 2 (n=15)	0.83 ± 0.03 (n=15)	0.82 ± 0.05 (n=15)
DAY 5-8	2.15 ± 0.05 (n=7)	2.23 ± 0.06 (n=12)	61 ± 1 (n=13)	63 ± 2 (n=15)	0.83 ± 0.02 (n=13)	0.89 ± 0.06 (n=15)
DAY 10-14	1.98 ± 0.09 (n=8)	2.25 ± 0.04 (n=8)	57 ± 2 (n=14)	64 ± 1* (n=10)	0.83 ± 0.02 (n=14)	1.07 ± 0.11* (n=10)
DAY 15-27	2.01 ± 0.06 (n=4)	2.37 ± 0.15 (n=2)	58 ± 2 (n=9)	67 ± 13 (n=2)	0.86 ± 0.04 (n=9)	1.27 ± 0.10 (n=2)

D_{AV} = Apparent diffusion coefficient averaged over three main orthogonal directions. All values are shown as mean±SEM. * p<0.05 (Student's t-test).

List of abbreviations

CSI	chemical shift imaging
D _{AV}	apparent diffusion coefficient averaged over three main orthogonal directions
dDNP	dissolution dynamic nuclear polarisation
DMEM	Dulbecco's modified eagle medium
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
FDG-PET	¹⁸ Fluoro-2-deoxyglucose positron emission tomography
FLASH	fast low angle shot
FOV	field of view
GCV	ganciclovir
hPGK	human phosphoglycerate kinase
HSV-TK	Herpes Simplex virus-1 thymidine kinase
HSV-TK/GCV	Herpes Simplex-1 thymidine kinase / ganciclovir therapy
i.p.	intraperitoneal
LV	lentiviral
MRI	magnetic resonance imaging
MRS	magnetic resonance spectroscopy
MOI	multiplicity of infection
MTS	(3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium)
NaOH	sodium hydroxide
PBS	phosphate buffered saline
p.i.	post-implantation
RARE	rapid imaging with refocused echoes
SEM	standard error of mean
TE	echo time
TR	repetition time
TRIS	tris(hydroxymethyl)aminomethane
WHO	World Health Organization

Figure 1

Figure 2

Figure 3

Figure 4