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LABORATORY INVESTIGATION

Evaluation of radiation-related invasion in primary patient-derived glioma cells and validation with established cell lines: impact of different radiation qualities with differing LET

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Abstract

Purpose Glioblastoma multiforme (GBM) is the most common primary brain tumor and has a very poor overall prognosis. Multimodal treatment is still inefficient and one main reason is the invasive nature of GBM cells, enabling the tumor cells to escape from the treatment area causing tumor progression. This experimental study describes the effect of low- and high-LET irradiation on the invasion of primary GBM cells with a validation in established cell systems.

Methods Seven patient derived primary GBM as well as three established cell lines (LN229, LN18 and U87) were used in this study. Invasion was investigated using Matrigel® coated transwell chambers. Irradiation was performed with low- (X-ray) and high-LET (alpha particles) radiation. The colony formation assay was chosen to determine the corresponding alpha particle dose equivalent to the X-ray dose.

Results 4 Gy X-ray irradiation increased the invasive potential of six patient derived GBM cells as well as two of the established lines. In contrast, alpha particle irradiation with an equivalent dose of 1.3 Gy did not show any effect on the invasive behavior. The findings were validated with established cell lines.

Conclusion Our results show that in contrast to low-LET irradiation high-LET irradiation does not enhance the invasion of established and primary glioblastoma cell lines. We therefore suggest that high-LET irradiation could become an alternative treatment option. To fully exploit the benefits of high-LET irradiation concerning the invasion of GBM further molecular studies should be performed.

Keywords Irradiation · Glioblastoma multiforme · Invasion · Survival · Primary cells · Brain tumor

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Introduction

Malignant gliomas are the most frequent and lethal cancers of the central nervous system. Of those, glioblastoma multiforme (GBM) is generally associated with the worst prognosis [1]. Despite technological advances in the current standard multimodal treatment regime including surgery, concomitant and/or adjuvant chemo- and radiotherapy (RT), GBM remains refractory and local progression-free survival is still a major problem [2]. The infiltrative behavior of the tumor limits surgical resection as it could cause loss of brain function [3]. Due to the impenetrable nature of the blood brain barrier chemotherapy is presently limited to temozolomide. RT is a highly effective component of multimodal treatment however dose escalation is limited even with advanced technologies as dose-escalation strategies have the potential to damage healthy, functional organs

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[4, 5]. Despite ongoing research to improve GBM therapy, the 5-year survival is below 10% and the overall survival of patients is very low (4–18 months) [6, 7]. High invasiveness and enhanced migratory capacity compared to other cancer types is a common feature of GBM [3]. While other aggressive tumors spread to distant organs, GBM rarely metastasize outside the brain. Due to GBM's active invasion, the tumor often recurs within 1–2 cm to the primary tumor [8]. Common features of GBM include the gain of motility and the expression of proteases to digest the extracellular matrix surrounding the tumor [9].

Irradiation with low linear energy transfer (LET) X-ray is a major component of GBM treatment. Since 1991, it is controversially discussed whether low-LET irradiation increases the invasive potential of GBM [10–12]. Up to 90% of the surgically removed primary GBM's recur in close proximity to the resected and irradiated tumor origin [13]. As the name GBM already reveals, this cancer type is a combination of several pathologically heterogeneous cells, exhibiting distinct characteristics like being dissimilar in morphology and proliferation [14, 15]. In the current study, a system to isolate GBM cells from patient-derived tumor tissue was established. With those primary GBM cells it was then possible to evaluate the patient-specific invasive potential.

Although RT using X-rays is still the standard of radiation treatment of GBM, more densely ionizing high-LET particles are discussed in this context [16–19]. In contrast to low-LET radiation which mostly induces DNA damage indirectly via the production of reactive oxygen or nitrogen species, high-LET particles interact mainly directly with the DNA. The higher LET results in complex and clustered DNA damage along the particles track through the cell nucleus as compared to the more simple and evenly spread DNA damage seen after low-LET radiation [20].

This study was performed to compare in detail the effects of low- and high-LET irradiation on the invasion of seven primary cell lines derived from patient tumor tissue as well as three established human GBM cell lines. The aim was to understand differential invasive potential and to develop future individualized treatment strategies.

Materials and methods

Primary cell isolation from patient-derived tumor tissue

Patients ≥ 18 years of age with primary GBM tumors were eligible for participation in the experimental RadGlio study. The trial was approved by an ethic vote of the ethics commission. Patient-derived tumor tissue was cut with a scalpel into small pieces and enzymatically digested for 30 min at 37 °C in a mixture consisting of DNase (2000 µ/ml, #LK003170, Worthington) and Papain (20 µ/ml, #LK003176, Worthington). Enzymatic activity was stopped with an Ovomucoid inhibitor (10 mg/ml, #LK003182, Worthington) at room temperature and an erythrocyte lysis was performed for further 20 min at room temperature. Primary GBM cells were seeded into a GelTrex or Matrigel coated plastic flask and cultivated in RPMI 1640 (R8758, Sigma) supplemented with 10% FCS (F7524, Sigma) and 1% penicillin/streptomycin (P0781, Sigma). Seven primary cell lines (T84, T76, H5, H19, H34, H45, H46) isolated from patient tumor tissue were included in this experimental study.

GFAP staining

In order to ensure that the freshly isolated tumor cells are of glial origin a glial fibrillary acidic protein (GFAP) staining was performed. 2×10^4 cells were seeded on a sterilized cover slip. The next day cells were fixed with 4% PFA for 1 h. The cells were washed twice in PBS, then with blocking buffer consisting of 1% BSA, 0.1% Triton X-100 and PBS. The primary antibody (GFAP rabbit anti human, DAKO #Z0334) diluted 1:500 in blocking buffer with the addition of 2.5% normal goat serum was incubated overnight. The secondary antibody (Alexa-488 anti-rabbit, life technologies #A11008) was diluted 1:500 in PBS and allowed to bind for 1 h. Following a final PBS washing step, Vectashield® (#H-1200, Vectorlabs) with DAPI was used to mount the microscope slide. Images were acquired using the Axioskop 2 plus (Zeiss).

Cell culture of established cell lines

Human established GBM cells were obtained from the University Hospital of Heidelberg. Cell line authentication was performed by Eurofins Genomics, Germany. LN229 and LN18 were cultivated in RPMI 1640 (R8758, Sigma) supplemented with 10% FCS (F7524, Sigma) and 1% penicillin/streptomycin (P0781, Sigma). U87 was maintained in DMEM supplemented with 10% FCS and 1% penicillin/streptomycin. Human fibroblasts, MC-IXC—purchased from ATCC—were cultured in a 1:1 mixture of EMEM and F12 Medium supplemented with 10% FCS and 1% penicillin/streptomycin. All cells were kept at 37 °C in a controlled atmosphere with 5% CO_2 and 95% humidity.

High-LET Irradiation

Cells were seeded 24 h prior to irradiation at a density of 4×10^5 cells per 10 cm^2 in a ring system to enable the irradiation at the alpha irradiation source. The ring system consisted of two metal rings (Ø 3.8 cm) between which a 4.7 µm polypropylene foil was stretched. The foil ensured that the energy loss of the alpha -particles is low enough

that the remaining range of the particles is enough to traverse a monolayer of cells. For increased cell attachment the foil was coated with Cell Tak (1 µg/cm^2, FALC354241, Corning).

High-LET α-particle irradiation was performed using an especially designed cell irradiation platform [21], which uses Americium-241, emitting α-particles with an energy of 5.49 MeV (LET = 85 keV/µm). An activity of 0.37 GBq and an energy of the particles at the cell surface of 2.7 MeV (LET = 146 keV/µm) caused a dose rate of 0.12 Gy/min. The additional dose of secondary X-rays, applied to the cells, is 10^{-4} of the α-particle dose and can therefore be neglected. The fluence of the α-particles was determined by irradiation and etching of CR39 plastic nuclear track detectors and resulted in $(4.68 \pm 0.03)\ 10^{-3}$ $\text{min}^{-1}\mu\text{m}^{-2}$. Applying a dose of 1.3 Gy leads to (50.7 ± 0.4) $10^{-3}\ \mu\text{m}^{-2}$. Measurements of cell size showed a mean nucleus size of ~ 150 µm^2, which causes 7.60 ± 0.06 hits per nucleus, according to Poisson statistics. Thus, 94% percent of cell nuclei receive a dose of at least 0.5 Gy whereas 0.5% of the cell nuclei are not hit at all.

Low-LET irradiation

Low-LET X-ray irradiation was performed either using the RS225A irradiation device (Gulmay/Xstrahl, UK) at a dose rate of 1 Gy/min (15 mA, 200 kV) or a clinical linear accelerator (ONCOR, Siemens) at a dose rate of 3 Gy/min (6 MV photons with 2 cm water-equivalent build-up). As both sources deliver low-LET photon irradiation (LET < 2 keV/µm) the radiobiological differences are very small and could be neglected. Radiation with the RS225A could be performed with the ring system described before. For irradiation at the clinical linear accelerator conventional cell culture flasks were used.

Colony formation assay (CFA)

Cells were irradiated with different doses (X-ray: 0, 1, 2, 4, 6 and 8 Gy; alpha particles: 0, 0.1, 0.2, 0.4, 0.8, 1.6 and 3.2 Gy). Five rings were irradiated per dose. After irradiation cells were trypsinized and cells from each ring were plated into three 12-well plates, so that 180 wells were evaluated per dose. 12 days after plating, the colonies were fixed with ice-cold methanol, stained with 0.1% crystal violet and counted. Colonies consisting of more than 50 single cells were counted as one colony with the GelCount™ (Oxford Optronics). Survival curves were fitted according to the linear quadratic model (LQ) with the equation $\ln \text{SF} = -\alpha \times \text{D} - \beta \times \text{D}^2$ by the Origin Software (OriginLab). Each CFA was performed twice.

Invasion assay

After irradiation the standard medium was exchanged with medium containing 0.5% FCS (serum starvation). 24 h after irradiation invasion assays were started with 2×10^4 cells per insert, seeded under serum starvation. Corning 24-well 8 µm pore sized transwell inserts uncoated as well as coated with matrigel were used according to manufacturer's instructions. After 24 h non-invading cells were removed, invaded cells were fixed and stained. Five independent microscope fields of each membrane were counted using a Zeiss Imager Z1m microscope at ×10 magnification. All invasion assays were performed at least in triplicate. The invasion was calculated as the mean number of cells invading through the matrigel coated membrane divided by the mean number of cells migrating through the uncoated membrane (control insert). Relative invasion of irradiated cells was normalized to sham irradiated cells.

Statistical analysis

Mean values were calculated and are presented ± standard error of the mean (SEM). Significance was evaluated by the Student's *t* test (GraphPad Prism). A p value of ≤ 0.05 was considered as statistically significant.

Results

Radiosensitivity of GBM cells after high- and low-LET irradiation

In order to determine the equivalent dose of alpha particle (high-LET) and X-ray (low-LET) irradiation, LN229 and LN18 cells were irradiated with X-rays and alpha particles and the clonogenic survival was measured. The plating efficiency of LN229 respectively LN18 was approximately 56%. As shown in Fig. 1, both cell lines show a similar radiosensitivity after X-ray as well as after alpha particle irradiation. While, as expected, X-ray survival curves depict the typical shoulder, cell survival after high-LET irradiation shows a lower cell survival per dose.

Based on the LQ fit the equivalent dose to 4 Gy X-ray irradiation was calculated as 1.3 Gy alpha particle irradiation in the CFA. Therefore, 1.3 Gy was applied in all other assays as equivalent dose to 4 Gy X-ray irradiation.

X-ray but not alpha particle irradiation increases invasion of human glioblastoma cells

The effects of X-ray and alpha particle irradiation on the invasion of the three established GBM cell lines, LN229, LN18 and U87 were investigated by a matrigel transwell

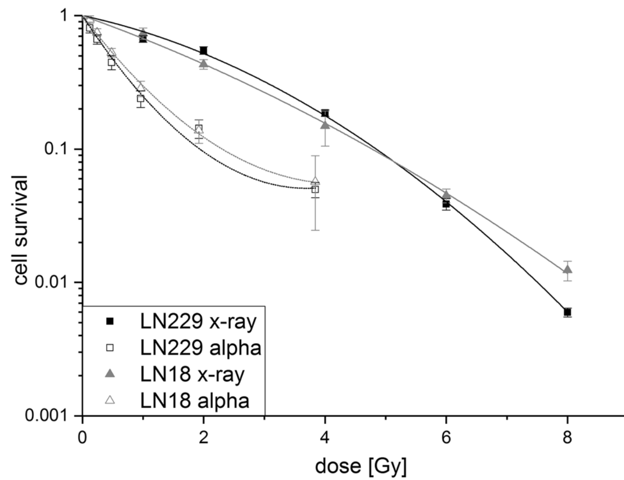

Fig. 1 Survival curves after X-ray and alpha particle irradiation. Cell survival curves of two established glioblastoma cell lines, LN229 and LN18, after X-ray and alpha particle irradiation were fitted to the linear-quadratic-model. While X-ray survival curves show the typical shoulder, cell survival after high-LET irradiation exploits lower cell survival per dose. Mean values of 2 biological replicates with each 180 replicates per dose and the SD are presented

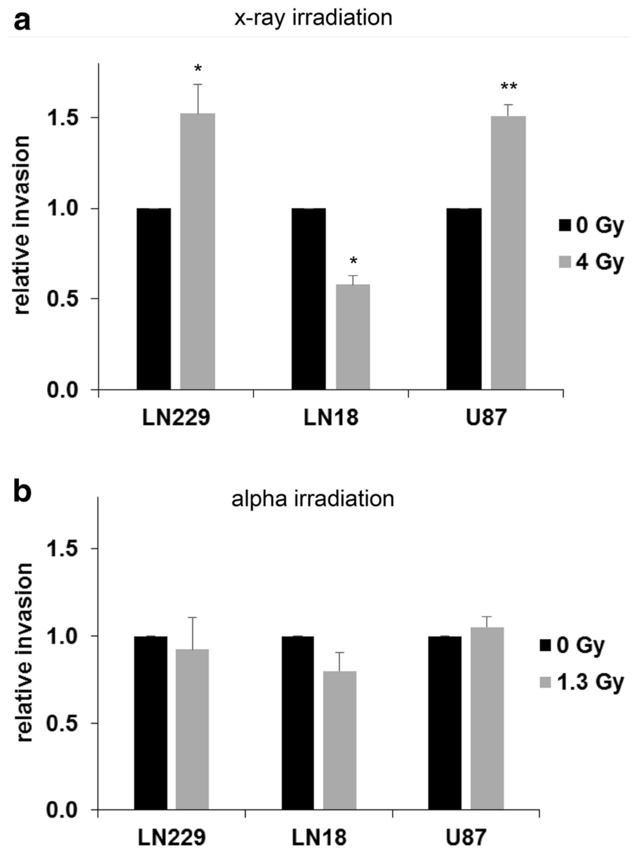

Fig. 2 Invasion assay with established cell lines after irradiation. Relative invasion of 3 GBM cell lines, LN229, LN18 and U87, 24 h after X-ray (**a**) and alpha particle (**b**) irradiation in relation to sham irradiated (0 Gy) cells. LN229 and U87 demonstrate a low-LET (**a**) but not high-LET (**b**) radiation enhanced invasion. Shown is the mean value and SEM of 4 (**a**) or 3 (**b**) replicates. A p value ≤ 0.05 is indicated as *, $p \leq 0.01$ as **

assay. A dose of 4 Gy X-rays affected the invasion of the established cells in a cell line dependent manner (Fig. 2a). 4 Gy X-ray irradiation induced a significant increase (1.5-fold) in LN229 ($p \leq 0.05$) and U87 ($p \leq 0.01$), whilst the invasion of LN18 significantly decreased (0.6-fold, $p \leq 0.05$). As Fig. 2b shows, the invasion of all three tested established cell lines remained unaffected by alpha particle irradiation.

Patient-derived primary cells are of glial origin

In order to investigate the effect of high- and low-LET irradiation on primary GBM, GBM cells were isolated from patient derived tumor material directly after surgery. To prove the glial origin the isolated tumor cells were stained for GFAP (glial fibrillary acidic protein) by immunofluorescence staining. As shown in Fig. 3 all isolated primary cells express GFAP as indicated by the green staining. Cells could therefore be used for further experiments.

Primary GBM cells show high basal invasive phenotype

GBM is considered as a very invasive tumor entity. Primary GBM cells were therefore tested for their basal invasive potential compared to brain fibroblasts (CRL) by matrigel transwell assays. We found that in comparison to the control brain fibroblasts all tested GBM cell lines showed an increased invasiveness (Fig. 4). T84 was 2.6-fold (± 1.1), T76 3.4-fold (± 0.6, $p \leq 0.05$), H5 twofold (± 0.6), H19 3.1-fold (± 0.3, $p \leq 0.01$), H34 twofold (± 0.6), H45 4.4-fold (± 1.1, $p \leq 0.05$) and H46 1.3-fold (± 0.1, $p \leq 0.05$) as invasive as the control fibroblasts.

Primary GBM cells confirm low-LET but not high-LET enhanced invasion

To prove the radiation-induced effects seen in the established cell lines, primary GBM cells were irradiated and the invasion was determined by matrigel transwell assays. As already observed in the established cell lines, the invasion of four primary GBM cell lines increased significantly after 4 Gy X-ray irradiation: T84 1.4-fold (± 0.1, $p \leq 0.05$), T76 1.4-fold (± 0.1, ≤ 0.05), H34 1.7-fold (± 0.2, $p \leq 0.05$) and H45 1.6-fold (± 0.2, $p \leq 0.05$) (Fig. 5a). Except for H19 the other primary GBM cell lines showed an increasing tendency: H5 1.5-fold (± 0.3) and H46 1.4-fold (± 0.2). The invasion of one primary GBM cell line, H19 (1.1-fold ± 0.05), remained unaffected after X-ray irradiation.

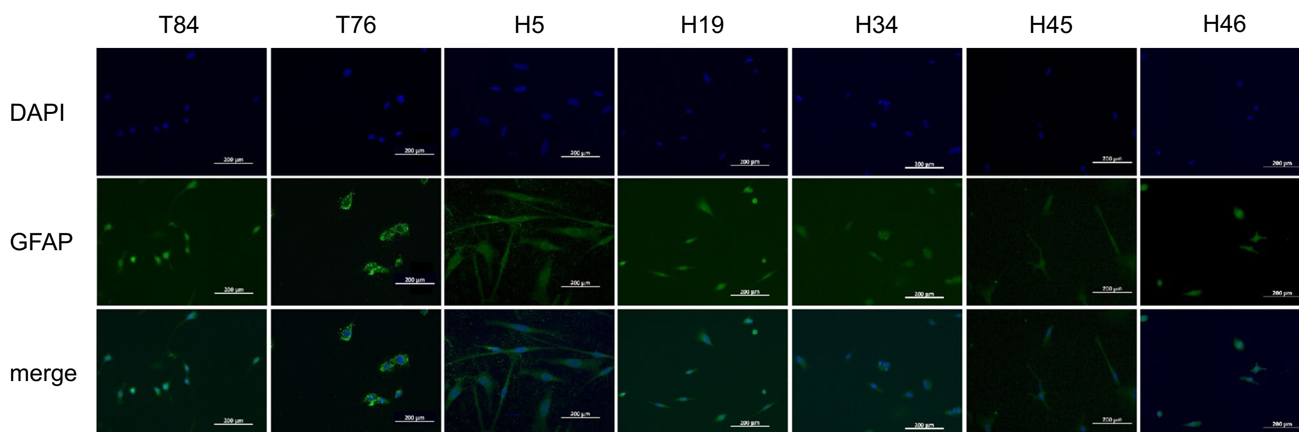

Fig. 3 GFAP staining of patient-derived GBM cells. Shown are exemplary pictures of the primary GBM cells stained with GFAP antibody and anti-rabbit Alexa 488. Green signal shows GFAP expression and indicates glial origin. Scale bar represents 200 µm

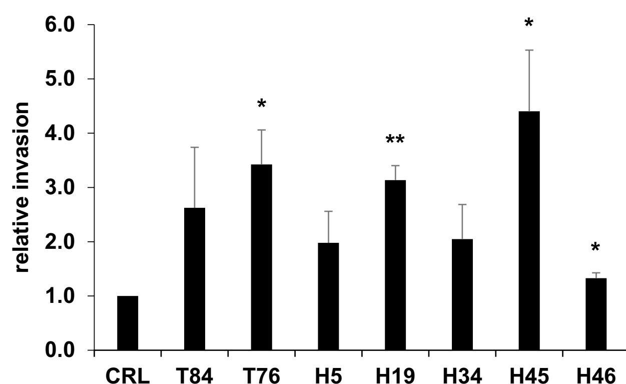

Fig. 4 Basal invasion of primary GBM cells. Shown is the basal invasion of the primary GBM cells in relation to brain fibroblasts (CRL). All tested primary cells show increased aggressiveness in comparison to the fibroblasts. The bar consists of the mean value of 3 replicates and the SEM. A p value ≤ 0.05 is indicated as *, $p \leq 0.01$ as **

Irradiation with the equivalent dose of alpha particles (1.3 Gy) did not affect the invasion of the primary GBM cell lines (Fig. 5b), confirming the data obtained with established cell lines.

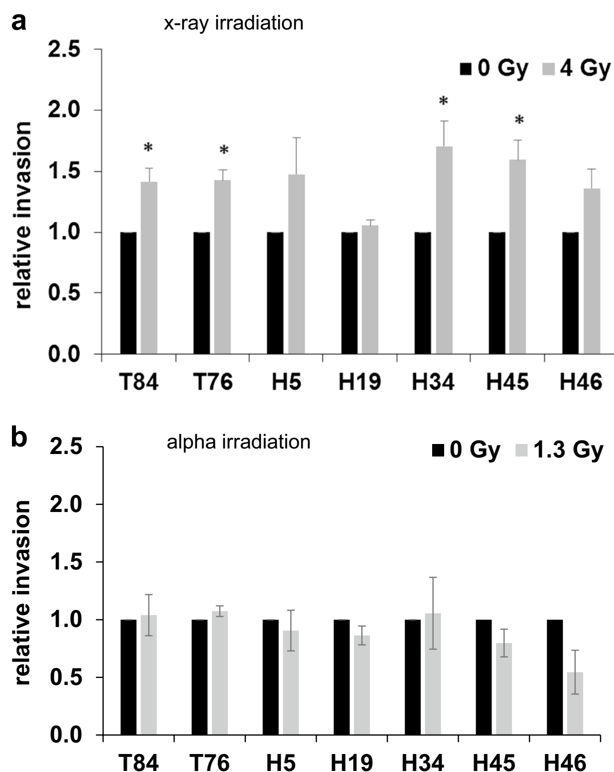

Fig. 5 Invasion assay with primary GBM cells after irradiation, showing the relative invasion of 5 primary GBM cell lines 24 h after X-ray (**a**) and alpha particle (**b**) irradiation in relation to sham irradiated (0 Gy) cells. All but one primary cell line demonstrate low-LET (**a**) but not high-LET (**b**) enhanced invasion. Shown is the mean value of 3 replicates with their SEM. A p value ≤ 0.05 is indicated as *

Discussion

In the present RadGlio study, primary GBM cell cultures from patient tissue were successfully established. It is the first study investigating the invasive behavior of seven primary patient-derived GBM cells after low- and high-LET irradiation. The isolated primary GBM cells are naturally far more invasive than control brain fibroblasts, indicating their high aggressiveness. Interestingly, the invasive potential differed significantly within cell lines, reflecting a high heterogeneity.

We could show that all but one of the primary GBM cell lines exhibited a radiation-enhanced invasion after low-LET

irradiation, which is in line with findings by Baulch et al. [22]. The observed radiation-induced invasion differed inter-individually between cell lines. This is confirmed by our data using established cell lines, demonstrating that X-ray irradiation with 4 Gy significantly increased the invasion of LN229 and U87 but not of LN18 cells. Likewise Park et al. showed that the invasion of U87 (PTEN mutated) was enhanced whereas invasion of LN18 (PTEN wt) was not influenced by low-LET irradiation [23]. Cordes et al. found that the invasion of LN18 was not changed by low-LET irradiation, confirming our data [24]. In contrast to our results, they showed that the invasion of LN229 is not affected by X-ray irradiation. This might be due to different applied irradiation doses or the time cells were exposed to serum starvation. Rieken et al. demonstrated a low-LET radiation-induced migration of U87 and LN229, supporting our results that irradiation increased aggressiveness [11].

In contrast to low-LET irradiation, our results clearly demonstrate that high-LET irradiation with alpha particles does not cause any changes in the invasive potential of established as well as primary GBM cell lines. This is well in line with previously published data revealing that the invasion of U87 is not increased after high-LET irradiation [25]. In addition Akino et al. [26] and Ogata et al. [27] could prove that high-LET irradiation did not cause disadvantageous effects on the invasive potential of lung cancer cells.

As today basically all GBM patients are treated with low-LET RT [28]; avoidance of RT-related invasion might be a potential approach to reduce treatment failures. However, not all evaluated GBM cell lines exhibited radiation-enhanced invasion indicating that tumor heterogeneity is an important topic for personalized medicine [29]. For future individualized GBM tumor treatment it may therefore be essential to identify those tumors, which become more invasive after low-LET irradiation. Several RT approaches could be followed: For one, target volumes may have to be increased in tumors exhibiting high invasive potential; alternatively, higher doses per fraction may be a good solution to prevent low-dose triggered invasion. Another strategy could be the application of high-LET radiotherapy, since it did not affect the invasion. During the last decade, high-LET irradiation has already been suggested as an improved cancer treatment option due to the sparing of healthy tissue and the superior biophysical characteristics. Recently the benefits of high-LET irradiation on GBM progression have been shown in several in vitro studies using established cell lines [16, 30, 31]. Most importantly, there are data hinting that there might be an advantage of high-LET irradiation for glioblastoma patients however, to date results from clinical trials confirming this idea are still lacking [17, 18, 32]. It is notable to say that some centers offering high-LET radiotherapy have refrained from treating glioma patients, and have focused on other tumor entities. Reasons for this are currently under discussion, but certainly reflect the complexity of high-LET RT and the urgent necessity for further preclinical radiobiological research.

In summary, an isolation system for primary GBM cells from patient-derived tumor tissue was established and by exposing those primary cells to different radiation qualities, an evaluation of the patient-specific tumor invasion was possible. Even though the clinical validation is still missing, our in vitro findings indicate that low-LET irradiation enhanced invasion might contribute to recurrences after conventional photon therapy. As none of the evaluated GBM cell lines showed high-LET radiation-induced invasion we suggest high-LET irradiation might have the potential for personalized treatment concepts for those patients that might suffer from low-LET increased invasion. Alternatively, must be discussed that this effect of high-LET RT might also be achieved by higher doses per fraction prescribed to adequate target volumes [33].

As the highly invasive potential of GBM cells enables the tumor to escape the treatment and thereby causes tumor progression, examining the invasive genotype and pathway signaling may help identifying molecular markers for GBM treatment. In conclusion, our results show an urgent need for further molecular studies identifying novel biomarkers that can predict the invasive response to irradiation and subsequently enable individualized treatment concepts.

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Data availability The datasets generated during and analysed during the current study are available from the corresponding author on reasonable request.

Compliance with ethical standards

Conflict of interest Every author of this manuscript declares that he/she has no conflict of interest.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. This article does not contain any studies with animals performed by any of the authors.

Informed consent Informed consent was obtained from all individual participants included in the study.

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