ATATÜRK ÜNİVERSİTESİ YAYINLARI
ATATURK UNIVERSITY PUBLICATIONS

Research Article DOI: 10.62425/jlasp.1704777

The Effect of Flattened and Unflattened Beams on Apoptotic, Tumor Suppressor, and Cell Proliferation Gene Expression Levels in Preclinical Malignant Melanoma Radiotherapy

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ABSTRACT

The current evidence base remains lacking regarding the differences in radiobiological outcomes dependent on the presence of a flattening filter in malignant melanoma (MMe) radiotherapy. We designed the present study to elaborate on the malignancy-related gene expression profiles in MMe tumors in response to flattening filter (FF) and flattening filter-free (FFF) beams. Twenty-eight NOD-Prkdc IL2rg tm1 (NSG) mice were randomly assigned to four experimental groups. The non-engrafted Control and MMe groups did not receive irradiation, while the FF-400 and FFF-1400 groups received single-dose 20 Gy ionizing radiation (IR) in FF (400 MU/min) and FFF modes (1400 MU/min). B-cell lymphoma 2 (BCL-2), Bcl-2-associated x protein (BAX), phosphatase and tensin homolog (PTEN), marker of proliferation Ki-67 (Ki-67), and tumor necrosis factor alpha (TNF-α) gene expression levels were evaluated 48 hours post-IR. All tested genes were significantly upregulated in the MMe group compared with those in the Control group ($p < .001$). Expressions of BAX, PTEN, and TNF-α were significantly higher ($p < .001$) in the MMe-IR groups, depending on the different dose rates. Significant differences in BAX and PTEN levels were found in response to FF and FFF beams ($p < .001$). Ki-67 and BCL-2 were decreased after IR ($p < .001$), with the FFF beam exerting a more prominent effect than the FF beam ($p < .001$). Our results indicate that both FF and FFF beams effectively induce radiobiological changes regarding apoptosis, tumor suppression, and cell proliferation processes, while the FFF beam is superior to the FF beam in MMe.

Keywords: Dose rate, Gene expression, Ionizing radiation, Laboratory animals, Malignant melanoma.

Received Date	:25.05.2025
Revision Requested Date	:24.06.2025
Last Revision Date	:12.08.2025
Accepted Date	:07.10.2025
Publication Date	:03.02.2026

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Cite this article: Sümer, E., Aras, S., Kul Köprülü, T., & Tuncer, A. (2026). The Effect of Flattened and Unflattened Beams on Apoptotic, Tumor Suppressor, and Cell Proliferation Gene Expression Levels in Preclinical Malignant Melanoma Radiotherapy. *Journal of Laboratory Animal Science and Practices, 6*(1), 1-8. https://doi.org/10.62425/jlasp.1704777

Introduction

Malignant melanoma (MMe) is a type of skin cancer that arises from melanocytes located in the basal layer of the skin. Despite being a rare form of skin cancer, MMe has rising incidence and mortality rates and accounts for 75% of skin cancer-related deaths. Chemotherapy, radiotherapy, and interferon applications are treatment options for this type of cancer (Tímár & Ladányi, 2022). Radiotherapy is commonly used for various types of cancer, with over 50% of patients receiving it as part of their treatment plan (Gul, 2024; Gul & Duzova, 2024), and it is commonly applied for the treatment of MMe, despite the fact that these tumors are radioresistant and require the application of high doses (Tagliaferri et al., 2022). Modern technologies, such as intensity modulated radiotherapy (IMRT) and volumetric arc therapy (VMAT), can deliver a lethal dose of radiation to tumors

while minimizing damage to critical organs at risk (Demir et al., 2022; Kamer et al., 2023). This is achieved by organizing radiation fields spatially and temporally through changes in dose rate and beam shape. Utilization of flattening filter-free (FFF) beams in stereotactic body radiotherapy (SBRT) and stereotactic radiosurgery (SRS) is generally superior to conventional flattening filter (FF) beam-dependent techniques in terms of reduced treatment time and normal tissue toxicity, as well as optimal dose coverage (Aras et al., 2021). Nevertheless, the radiobiological effects of these beams on different cancers should be fully elucidated for the planning of an optimal management strategy and achieving better treatment outcomes.

While the effects of FFF beams have been investigated in several cancers (Nakano et al., 2018; Oktaria et al., 2017), to the best of our knowledge, their effect on MMe cell survival, significance, and the underlying molecular mechanisms are yet to be elucidated in vivo. Therefore, this preclinical comparative study was aimed at revealing the changes in the expression levels of key apoptotic, tumor suppressor, and cell proliferation genes in MMe tumors with respect to the presence of FF.

Methods

Study design

Twenty-eight adult male NOD-Prkdc IL2rg tm1 (NSG) mice were obtained from the Animal Experiments Laboratory of our institution. The animals were housed in non-pathogenic laminar flow cabinets and HEPA-filtered rooms using an individually ventilated caging system under standard laboratory conditions at 21 ± 2℃, 50 ± 10% humidity, and a 12-hour light/dark lighting cycle. An automated system routinely controlled room humidity, ventilation, and temperature. The experimental mice (weighing 20–30 g) were randomly assigned to the following experimental groups: Group 1 (n=8), consisting of MMe models without irradiation (Group 1); ionizing radiation (IR) groups, which received single-dose 20 Gy IR at an FF-based low dose rate (Group 2) and an FFF-based high dose rate (Group 3) (400 MU/min and 1400 MU/min, respectively; n=8 each); and Control (Group 4), which was not subjected to tumor formation or IR (n=4). This experimental study was conducted after obtaining approval from the Local Ethics Committee of Yeditepe University Faculty of Medicine Experimental Research Center (Date:27.03.2023, No: 2023/03-06B).

B16-F10 melanoma cancer cell line

The MMe cell line B16-F10 was obtained from the American Type Culture Collection (USA, CRL-6475™). The cells were incubated in Dulbecco's Modified Eagle's Medium High Glucose (DMEM-HG; Gibco™) containing a final concentration of 2% penicillin/streptomycin (Sigma-Aldrich) and 10% fetal bovine serum (Sigma-Aldrich) with humidified air at 37℃ and 5% CO_2. After reaching an 80% occupancy rate in cell culture flasks, the old culture media was removed, and the cells were rinsed using PBS. The cells were detached using trypsin (Sigma-Aldrich) and counted to prepare the mixtures for tumor inoculation.

MMe NSG mice models

For each animal, a total of 5×10^6 B16-F10 cells were prepared for tumor inoculation in a DMEM-HG-Matrigel mixture at a 1:1 ratio (Corning® Matrigel® Matrix, 356255), and subcutaneous injection of the mixture (100 µL per animal) was performed bilaterally at two points on the dorsal regions of NSG mice. Tumor growth was monitored until euthanasia, which was carried out via cervical dislocation on day 20 following inoculation. The isolated tumor tissues were then subjected to gene expression analyses.

Irradiation procedure

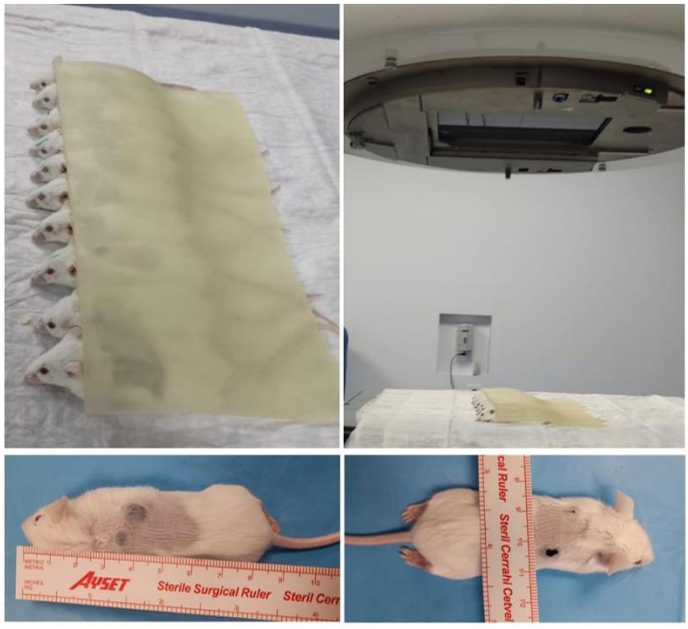
IR was performed under general anesthesia induced by intraperitoneal administration of ketamine 60 mg/kg and xylazine 8 mg/kg. Mice were placed in a supine position on a Plexiglass tray. To compensate for dose depth and distribution and increase the skin dose, a 10-mm bolus was placed on the radiotherapy field. The dorsal region was irradiated (single dose of 20 Gy) with FF-low dose rate (400 MU/min) and FFF-high dose rate (1400 MU/min) using a Trilogy linear accelerator (Varian Medical Systems, Palo Alto, CA, USA) (Figure 1).

Euthanasia procedure

At 24 hours following IR, animals were anesthetized via intraperitoneal ketamine (60 mg/kg) and xylazine (8 mg/kg) and euthanized by cervical dislocation. MMe tissues were

then surgically removed and preserved for genetic examination.

Figure 1.
Radiotherapy setup procedure for experimental malignant melanoma NSG mice models.



RNA isolation

RNA was extracted from tumor tissues using the GeneJET RNA Purification Kit (Thermo Scientific, K0731). Briefly, 30 mg of sample from each tumor tissue was incubated with lysis buffer, treated with proteinase K, and centrifuged. Absolute EtOH was added to the supernatant in a fresh tube. The mixture was transferred to a filtered column for purification and centrifuged. After washing, RNA was eluted with dH_2O, and the concentration of each sample was measured using the Qubit 4.0 Fluorometer device. The samples were stored at -80°C until further use.

cDNA synthesis and real-time quantitative polymerase chain reaction (RT-qPCR)

cDNA was synthesized using the iScript cDNA Synthesis Kit (Bio-Rad). A mixture of iScript Reaction Mix and Reverse Transcriptase was prepared, and the required amount of dH_2O and 1 µg of RNA template were added to the mixture. The prepared reaction tubes were incubated at 25℃ for 5 minutes, 46℃ for 20 minutes, and 95℃ for 1 minute, as per the manufacturer's instructions. Finally, the tubes were cooled down to 4℃.

RT2 qPCR Primer Assay and RT2 SYBR Green Mastermix (Qiagen) were used to reveal the expression profiles of the five targets: B-cell lymphoma 2 (BCL-2), Bcl-2-associated X protein (BAX), phosphatase and tensin homolog (PTEN), marker of proliferation Ki-67 (Ki-67), and tumor necrosis factor alpha (TNF-α). The prepared mixtures were distributed to the wells of a 96-well plate, including the relevant primers, in six replicates. The primer sequences and associated Tm values are shown in Table 1. Since the Tm temperature of each primer is different, each primer was studied separately. Finally, 2 ng of the cDNA templates were added to each well. GAPDH served as the housekeeping gene for normalization. Using Roche LightCycler 480 II, the reactions were initiated at appropriate temperatures. The results were analyzed using the GeneGlobe (Qiagen) tool.

Table 1.
Sequences and melting temperatures of primers used in real time polymerase chain reactions

Primers	Tm (°C)	Base Sequence
GAPDH Forward	60	5'AGGTTGTCTCCTGCGACTTCA3'
GAPDH Reverse	60	5'TGGTCCAGGGTTTCTTACTCC3'
BAX Forward	60	5'GGCGAATTGGAGATGAACTG3'
BAX Reverse	60	5'CAAAGTAGAAGAGGGCAACCAC3'
BCL-2 Forward	60	5'TCGCCCTGTGGATGACTGA3'
BCL-2 Reverse	60	5'CACTTGTGGCCCAGGTATG3'
TNF-α Forward	56.31	5'AAGCCTGTAGCCCACGTCGTA3'
TNF-α Reverse	57.38	5'GGCACCACTAGTTGGTTGTCTTTG3
PTEN Forward	60.5	5'TGTGGTCTGCCAGCTAAAGG3'
PTEN Reverse	60.5	5'AGGTTTCCTCTGGTCCTGGT3'
Ki67 Forward	55.88	5'CTGCCTGCGAAGAGAGCATC3'
Ki67 Reverse	53.83	5'AGCTCCACTTCGCCTTTTGG3'

Statistical analysis

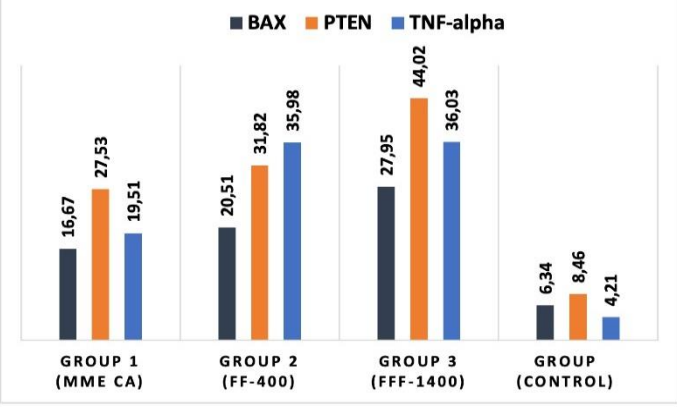
Data were analyzed using the IBM Statistical Package for the Social Sciences (IBM SPSS Corp., Armonk, NY, USA) 25 software package. An independent sample t-test was

performed to compare the mean ± SD values between different experimental groups. A two-tailed *p*-value < .05 was considered statistically significant.

Results

Gene expression levels of proapoptotic BAX, tumor suppressor PTEN, and inflammatory cytokine TNF-α were significantly higher in tumors of the MMe group than in normal skin tissues from the Control group (Figure 2 and Table 2). In the IR groups, these genes were significantly upregulated compared with those in the MMe group due to irradiation at different dose rates. In addition, the FFF-IR was more effective than the FF-IR in inducing BAX and PTEN expressions (Figure 2 and Table 2). The detailed comparisons between the groups with *p*-values are presented in Table 3.

Figure 2.
BAX, PTEN, and TNF-α gene expression levels in malignant melanoma.



Note: *BAX, Bcl-2-generated x protein; PTEN, phosphatase and tensin homologs; TNF-α, tumor factor alpha.*

Levels of the Ki-67 cell proliferation and anti-apoptotic BCL-2 transcripts were significantly higher in the tumors of the MMe group than in the Control group. Following a single dose of 20 Gy IR, the expressions of both transcripts were significantly reduced in both IR groups compared with those in the non-irradiated MMe group (Table 2 and Figure 3). Both Ki-67 and BCL-2 levels were significantly lower in the FFF-IR group than in the FF-IR group. Table 3 shows the p-values obtained from the comparisons between the groups.

Table 2.
Mean ± standard deviation values of BAX, PTEN, TNF-α, BCL-2 and Ki-67 genetic parameters obtained in the experimental NSG mice model.

	G1 MMe	G2 FF-400	G3 FFF-1400	G4 Control
BAX	16.66 ± 0.175	20.51 ± 0.117	27.95 ± 0.104	6.35 ± 0.100
PTEN	27.53 ± 0.302	31.82 ± 0.075	44.02 ± 0.251	8.46 ± 0.128
TNF-α	19.51 ± 0.060	35.98 ± 0.394	36.03 ± 0.376	4.21 ± 0.165
BCL-2	31.86 ± 0.406	25.59 ± 0.195	15.63 ± 0.176	5.82 ± 0.382
Ki-67	33.07 ± 0.356	27.59 ± 0.227	16.35 ± 0.309	6.91 ± 0.433

Note: Mean ± Standard Deviation, FF: Flattening Filter, FFF: Flattening Filter Free. BCL-2, B-cell lymphoma 2; BAX, Bcl-2-associated x protein; PTEN, phosphatase and tensin homolog; Ki-67, marker of proliferation Ki-67; TNF-α, tumor necrosis factor alpha.

Table 3.
p-values showing comparisons between the BAX, PTEN, TNF-α, BCL-2 and Ki-67 among the experimental groups.

Genetic parameters	G1 vs. G2 MMe vs. FF-400	G1 vs. G3 MMe vs. FFF-1400	G1 vs. G4 MMe vs. Control	G2 vs. G3 FF-400 vs. FFF-1400
BAX	< .001*	< .001*	< .001*	< .001*
PTEN	< .001*	< .001*	< .001*	< .001*
TNF-α	< .001*	< .001*	< .001*	.093
BCL-2	< .001*	< .001*	< .001*	< .001*
Ki-67	< .001*	< .001*	< .001*	< .001*

*Note: *Significance level was set at p < .05. BCL-2, B-cell lymphoma 2; BAX, Bcl-2-associated x protein; FF, flattening filter; FFF, flattening filter-free, MMe, malignant melanoma; PTEN, phosphatase and tensin homolog; Ki-67, marker of proliferation Ki-67; TNF-α, tumor necrosis factor alpha.*

Figure 3.
BCL-2 and Ki-67 gene expression levels in malignant melanoma. BCL-2, B-cell lymphoma 2; Ki-67, marker of proliferation Ki-67.

Discussion

The radiobiological effects of patient irradiation time and clinical dose rate on tumor cell survival have been discussed in the literature (Lowe et al., 2022). However, there is still a lack of sufficient in vitro and in vivo studies investigating the differential influence of a high dose rate using FFF beams. IMRT, VMAT, SRS, and SBRT techniques offer several advantages, including better conformal dose distribution in the target volume, improved dose homogeneity, and protection of adjacent normal tissues. While the radiobiological outcomes of SRS and SBRT have been investigated in detail (Kim et al., 2015), clinical dose rate-dependent effects and associated mechanisms remain poorly understood owing to factors such as radiosensitivity based on the cell cycle, cell type, doubling time, age, and histological type (Song et al., 2014).

The optimal dose rate for radiotherapy is believed to differ significantly depending on the type of tumor cells being treated. Both in vitro and in vivo radiobiological data indicated that different tumor types may exhibit varying responses to radiation at different dose rates, underscoring the importance of considering the clinical dose rate when planning cancer treatment (Ceylan et al., 2021; Dini et al., 2023). However, while the body of evidence regarding the differences in radiobiological responses to FF and FFF beams has been growing, the outcomes vastly differ between various settings. For example, Dubois et al. evaluated clonogenic survival in various cancer and normal cells and reported no differences between the outcomes of irradiation with FF and FFF beams (Dubois et al., 2015). Similarly, no differences in cell survival were reported regarding the effects of three different dose rates (500, 1000, and 3000 MU/min) on V79 fibroblasts and $FaDu_{DD}$ hypopharyngeal squamous cell carcinoma cells for doses ranging between 1 and 10 Gy (Sørensen et al., 2011). Further, no significant difference in cell survival was reported between V79 Chinese hamster lung fibroblasts and T98G and U87-MG glioblastoma cells in response to FF and FFF irradiations (Lasio et al., 2014). In contrast, Lohse et al. reported a reduction in T98G and U87-MG glioblastoma cell viability when irradiated with different dose rates ranging from 20 MU/min to 2400 MU/min. Using a radiobiological model, they proposed a dose-dependent increase in response to FFF beams in terms of cell death, particularly at 10 Gy and above (Lohse et al., 2011).

Some findings on different cell lines include the report of Karan et al., which reported comparable survival rates for V79 fibroblasts, H460 non-small lung carcinoma cells, and SiHa cervical carcinoma cells following acute irradiation with FF and FFF beams at 5 or 10 Gy (Karan et al., 2013). Also, the radiobiological effects of 600 MU/min FF and 2400 MU/min FFF beams were comparable for D384 astrocytoma, T98 malignant glioma, and SW1573 small cell lung cancer cells when irradiated with a single dose of 12 Gy (Verbakel et al., 2013). When the therapeutic effects of 420 and 2120 MU/min dose rates at 2, 4, and 6 Gy doses were compared, no differences were found in the responses regarding glioma stem-like cell viability, tumor sphere formation capacity, survival, cell cycle, apoptosis, or DNA damage (Hao et al., 2019). Laurent et al. investigated the anti-tumor immunological activity in CT26 murine colon cancer cells following 400, 1200, or 2400 MU/min FFF beams and failed to identify differences in the type I interferon pathway and immunogenic cell death indicators (Laurent et al., 2020). However, while many studies employing various doses, dose rates, and cell lines are unable to detect marked differences between the radiobiological responses, this may not apply to all settings. For example, Aras et al. found that laryngeal cancer responds differently to FF and FFF beams, as evidenced by histopathological findings and Ki-67 levels, exhibiting increased sensitivity to the latter (Aras et al., 2023).

Regarding melanoma, Bewes et al. (2008) reported a reduced cell survival for higher dose rates at a fixed total dose (Bewes et al., 2008). In agreement, a 2400 MU/min dose rate increased apoptosis in WC00046, WC00060, and WC00081 melanoma cells, suggesting a potential alternative therapy mode for melanoma. The treatment improved the survival of melanocytes while increasing the radiosensitivity and enhancing the apoptotic rate in melanoma cells (Sarojini et al., 2015). While these findings hold significance regarding future radiotherapy planning for melanoma, the results should be confirmed through preclinical and clinical studies demonstrating the radiobiological responses to FF and FFF beams. Therefore, in this study, we provide the next level of evidence for the differential effects of FF and FFF beams in melanoma radiation therapy. We investigated differences in the expression profiles of BAX, BCL-2, PTEN, Ki-67, and TNF-α induced by FF and FFF beams in an MMe mouse model, considering the radiobiological uncertainties in the literature regarding the dose rate effect.

BAX is a proapoptotic molecule that plays an important role in programmed cell death processes. The level of BAX is often altered in primary and metastatic melanoma cells, suggesting an impairment in the associated molecular mechanisms (Zeren et al., 2014). In our study, the BAX transcript level was significantly increased in response to IR with FF or FFF beams, suggesting that both irradiation modes were effective in promoting the apoptotic pathway in melanoma tumors. We also analyzed the levels of the BCL-2 transcript. The anti-apoptotic B-cell lymphoma 2 (BCL-2) protein inhibits the translocation of BAX to mitochondria during drug-induced apoptosis of cancer cells, resulting in the inhibition of this programmed cell death process (Murphy et al., 2000). Aligning with this phenomenon, the BCL-2 transcript levels were elevated in conjunction with BAX in the tumors of our MMe models, suggesting a means of evading apoptosis. Following irradiation with FF or FFF beams, BCL-2 was downregulated, and BAX was upregulated, exhibiting a significant shift in the BAX/BCL-2 ratio favoring the progression of apoptosis.

The tumor necrosis factor alpha (TNF-α) protein has been found to exert opposing effects in melanoma owing to its complex involvement in the regulation of both inflammation and apoptosis. While TNF-α may support tumor development and metastasis by altering the tumor microenvironment in some cancer types (Xie et al., 2025), its excessive levels may be helpful in suppressing tumor growth (Nenu et al., 2015). In this study, the TNF-α transcript was significantly upregulated in tumors that received IR. Importantly, TNF-α can activate BAX and inhibit BCL-2, promoting an increase in the apoptosis-favoring BAX/BCL-2 ratio. Considering the elevated BAX expression and the diminished BCL-2 expression found in the irradiated melanoma tumors, TNF-α upregulation appears to be a key factor involved in the beneficial radiobiological responses obtained in this study.

The tumor suppressor protein PTEN prevents the uncontrolled division and survival of cells, especially by inhibiting the phosphatidylinositol 3-kinase/AKT signaling pathway (Wang et al., 2020). In our study, we determined that the PTEN transcript was increased upon irradiation with FF and FFF beams, suggesting that these radiation therapies may help suppress the unregulated growth of MMe tumors.

Finally, we demonstrated significantly elevated levels of the Ki-67 transcript in melanoma tumors compared with those in controls, as well as a significant decrease in its levels after radiation. The Ki-67 protein is involved in cell division, and elevated levels of Ki-67 in cancer tissues typically indicate a more aggressive tumor (Ouh et al., 2024). Therefore, our findings imply that IR can effectively suppress melanoma cell proliferation in vivo.

A notable finding of our study was that high dose rate FFF beams were more prominent in inducing BAX and PTEN, as well as suppressing BCL-2 and Ki-67, than low dose rate FF beams, indicating that employing FFF beams in the clinical radiotherapy of MMe may help achieve better outcomes.

A limitation of this study was the analysis of acute-period gene expression. Thus, our future studies are directed at investigating subacute and chronic effects for different doses, dose rates, and cancer types. Moreover, in-depth studies are warranted to comprehend the fundamental mechanisms that contribute to the dose rate effects and to enhance the efficacy of radiation therapy. The evaluation should concentrate on the damage and repair processes in cancer cells caused by various radiation dose rates.

Additionally, this study included the analysis of early responses to different dose rates and did not incorporate changes in the downstream at the gene or protein level, such as caspase 3 activation. Thus, future studies should incorporate relevant western blot or immunohistochemical analyses to provide further mechanistic evidence. Finally, histopathological analyses are required to document morphological changes. This study highlights the significance of considering not only the physical radiation dose but also the radiobiological response when planning cancer treatment. Understanding the effects of different dose rates on cancer cell survival can enable radiation oncologists to make informed decisions, maximizing treatment efficacy and improving the clinical outcome of radiotherapy.

Conclusion

The FFF beam was more effective than the conventional FF beam in altering the gene expression profiles related to apoptosis, tumor suppression, and cell proliferation in MMe cells. The findings on genetic parameters regarding the in vivo clinical dose rate effect are expected to guide radiotherapy planning for MMe.

Ethics Committee Approval: This experimental study was conducted after obtaining approval from the Local Ethics Committee of Yeditepe University Faculty of Medicine Experimental Research Center (Date: 27.03.2023, No: 2023/03-06B).

Author Contributions: Concept - E.S., S.A.; Design - S.A.; Supervision – E.S., S.A.; Resources - T.K.K., A.T.; Materials – T.K.K.; Data Collection and/or Processing – E.S., S.A., T.K.K., A.T.; Analysis and/or Interpretation – E.S., T.K.K., A.T.; Literature Search – E.S., S.A., A.T.; Writing Manuscript – E.S., S.A., T.K.K., A.T.; Critical Review – S.A.

Peer-review: Externally peer-reviewed.

Funding: None.

Declaration of Interests: The authors declare that they have no competing interest.

Use of Artificial Intelligence: The authors declare that no artificial intelligence–based tools were used during the preparation of this manuscript.

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