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# Radiosensitivity of glioblastoma multiforme and astrocytic cell lines in cell signalling aspects

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## ABSTRACT

**Objectives:** The aim of this study is to investigate the radiosensitivity of Glioblastoma multiforme (GBM; U87 MG) and astrocyte (SVG p12) cell lines in vitro through the signalling pathways.

**Methods:** GBM and astrocytes were treated with 2, 4, 6, and 8 gray of ionized radiation, followed by a clonogenic assay. The effective dose of radiation was determined as 2 gray. Immunofluorescence technics selected to analyse the macrophage migration inhibiting factor (MIF), nuclear factor of activated T-cells cytoplasmic 2 (NFATc2), osteopontin (OPN), mammalian target of rapamycin (mTOR) and stage-specific embryonic antigen-1 (SSEA-1). Additionally, p53 and cell cycle assays were performed.

**Results:** On day 1, astrocytes showed decreased expression of MIF, OPN and mTOR and increased expression of SSEA-1 in the test group after 2 gray radiation. GBM showed decreased expression of p53 and mTOR, but increased expression of NFATc2. The results of MIF expression were found higher in GBM compared to astrocytes on day 1. Interestingly, on day 12, increased expression of SSEA-1, OPN and p53 were observed in both cell lines' test groups. Further analysis showed that all control groups of GBM and astrocytes were significantly accumulated in the S phase. After radiotherapy application, percentage of GBM in G0/G1 phases and especially in G2/M phases increased; conversely, in the S phase it decreased. Moreover, percentage of astrocytes increased in the S phase and decreased in G0/G1 phases and in G2/M phases.

**Conclusions:** This combination of findings suggests that as a result of the radiotherapy effect, GBM started to accumulate on check points. The central question in this study focused on changes in molecular protein expression in cancer cells after radiotherapy, particularly key signalling pathways of tumorigenesis and a new possible point of view for treating such diseases.

**Keywords:** Glioblastoma multiforme, cell cycle, radiosensitivity, cell signalling

Gliomas are the most common primary intracranial tumour type in adults, representing 81% of malignant brain tumours. Glioblastoma multiforme (GBM) is the most aggressive type of glioma, with average of 14 months survival time even after surgical operation, chemotherapy and radiotherapy treatments [1].

Traditionally, GBM had been divided into two

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groups as “primary-de novo” and “secondary". However, a new molecular staging system was reported by WHO in 2016 and molecular .classification of the tumour characteristics. This molecular classification indicated that IDH wild type glioblastoma develops faster and more aggressively than IDH mutant variants, which are more common in elderly people. The definition of new GBM subtypes implicates that tumours differences are not only cytogenetics origin but also in histopathological differences [2].

The current standard treatment procedure for early-diagnosed GBM patients is surgical resection followed by adjuvant radiotherapy and concomitant temozolomide chemotherapy with 60 Gray (Gy) (conventionally divided into fractions) [3]. The use of radiation in the brain has significantly acute adverse effects such as permanent radiation damage, fatigue, hair loss and increased intracranial pressure [4]. Tumour is heterogeneous group which includes cells that have stem cell like character. These cells may be increase with differentiated phenotype. [5]. For that reason, current clinical treatments can develop the tumour initiator cell (TIC) subpopulation and may cause tumour recurrence due to self-renewal characteristics. TICs or stem cells in tumour tissue [6] also stimulate the cancer progression, consequently, altering signalling pathways and cell cycle regulation [7]. Since tumours have various types of molecular subgroups, cell identification of tumour initiator cells has always been the missing puzzle pieces in the approaches to cancer initiation. Identification of specific cell types by cell surface markers such as the stage-specific embryonic antigen-1 (SSEA-1) and osteopontin (OPN) is one of the most reliable ways of selection and isolation of these cells [8].

A number of researchers have reported that SSEA-1 / CD15 / Lewis X, a neural progenitor cell marker, is expressed in GBM cancer stem cells. In addition to their regeneration and differentiation ability, SSEA-1 positive cells give much higher rise to tumour initiation in mouse xenograft models compared to SSEA-1 negative cells [9]. The main target of radiotherapy and chemotherapy is to minimize the high proliferative cells, ending up with the slow-cell cycle and silent stem cells [10]. The cell cycle is the fundamental function for properly duplicating DNA [11]. Previous research has shown that p53, one of the basic proteins of the control points in the cell cycle, mediates transition from G1 phase to S phase and from G2 phase to mitotic division with other regulatory proteins in radiation-induced DNA damage [12]. Among all tumour suppressor genes, p53 plays an important role in the pathogenesis of many common malignancies including brain cancer. p53 has been shown to induce apoptosis, activate cell cycle, stimulate cell differentiation, and involve tumour suppressor activity, including in DNA repair pathways [13]. On the other hand, it has been shown that the increase of p53 expression is less in radiation-resistant cell lines and based on these observations, these investigators have argued about how function of p53 is essential for radiation sensitivity [14].

Mammalian target of rapamycin (mTOR) is a mediator protein with a key role in the phosphatidyl-inositol-3-kinase (PI3K) signalling pathway, has an important role in the regulation of biological processes such as cell growth, proliferation and cell survival. Abnormal signalling in mTOR / PI3K signal is marked in many types of cancer and may affect tumorigenesis and resistance treatment.

Macrophage migration inhibiting factor (MIF) is a mediator protein and effective as a cytokine, hormone and enzyme [15]. When MIF functions as a cytokine, it specifically induces angiogenesis and cell cycle, besides inhibits p53-induced apoptosis and plays a significant role in tumorigenesis by activating PI3K / Akt pathway [16].

Osteopontin (OPN or SPP1) is expressed in many cell types but especially in osteoblasts, osteocytes, chondrocytes, fibroblasts, macrophages and T cells. Furthermore, OPN is an early stage differentiation marker for osteoblasts and osteoclasts [17]. This protein is a pro-inflammatory and largely associated with cancer pathophysiology, cell adhesion, migration, tumour progression, metastasis development and resistance to treatment [18]. GBM patients have positive association between OPN expression and malignancy grade besides OPN serum level was a poor prognostic marker for GBM patients [19].

Nuclear Factor of Activated T-Cell (NFAT) family members, first described as a transcription activators of T cells, play roles in many biological processes such as inflammatory response, angiogenesis, cardiac valve formation, skeletal development, bone homeostasis, axonal orientation [20].

To achieve a better description of GBM tumour

cells and astrocytic cell line biology in the view of tumour response to radiation treatment, OPN, MIF, nuclear factor of activated T-cells cytoplasmic 2 (NFATc2) for inflammation, mTOR for autophagy, p53 for cell cycle, SSEA-1 for tumour initiating futures were investigated with a considered cell signalling approach.

## METHODS

### Cell Culture

GBM (U87 MG ATCC® HTB14™) and astrocyte (SVG p12 ATCC® CRL-8621™) cell lines were cultured in 10% Fetal Bovine Serum (Gibco-42F957/K) containing Eagle's Minimum Essential Medium (Sigma-RNBG0666). Cells were passaged every 2-3 days after confluence reached about 80%. Cells were cultured and used between passage numbers of 4-10. cell counting was performed with cell count and viability kit (Muse Cell count & viability kit Millipore-2932688). According to the viable cell number, solutions were diluted to 5×104 cells/mL and used in experimental culture technic. Cells were cultured on the 15 mm cover glasses. Each well of 6 well plates contained three 15 mm cover glasses.

### Irradiation

For full scatter conditions, a special type of solid water phantom was designed, and 6 well-plates were placed in, along the central axis. 6 well plate was filled with culture medium and placed in the phantom. Total depth of cells was set to be 1.5 cm from the couch top. The set-up was scanned with a Toshiba Asteion (Japan) CT. For achieving the monitor units (MU) of prescribing doses (including attenuation of the couch), a RT plan which ensures uniform dose on cells was created by Xio TPS (v4.8, Elekta, Sweden); gantry angle of 180°, at 100 cm source to surface distance (SSD) to the couch top, using a 23×23 $cm^2$ field size at 1.5 cm depth.

Irradiation was performed using 6 MV Elekta Precise linac (Elekta, Sweden) at the conditions of RT plan setup described above. Dosimetry verification was evaluated by ion chamber for absolute dosimetry and by calibrated Gafchromic EBT3 (NJ, USA) films which were cut in the shape of flasks and placed at the bottom of them for ensuring uniform dose. Measurements showed that doses were accurate to within ±3%. Cells were then irradiated with various doses (2, 4, 6, 8 Gy) at a dose rate of 300 MU/min (Fig. 1). The control group was also transferred to Ege University Department of Radiation Oncology but left non-irradiated, to expose the whole cell groups to the same environmental conditions. The irradiated and control group then assayed for colony formation.

### Clonogenic Survival Assays

Exponentially growing cells were cultured and plated in 6 well dishes. To plate the accurate number of cells is essential for obtaining the correct data for plating efficiency (PE). Cells were left to grow in humidified $CO_2$ incubator to form sufficiently large clones consisting of 50 or more cells. At 12$^{th}$ day of colonization, colonies were stained with crystal violet dye (Merck 42555) and colonies containing ≥ 50 cells were scored (Fig. 2). Each colony represented one cell surviving after irradiation or without irradiation for the control group. The whole procedures were repeated three times independently. Clonogenic survival curves were plotted as the log of the surviving fraction as a function of the dose.

Plating efficiency (PE) was given by Equation 1 [21].

Equation 1 PE (%) = Number of colonies counted × 100 / Number of cells seeded

The cell survival fraction (SF) was calculated by Equation 2 [21].

Equation 2 SF = Colonies counted / Cells seeded × PE.

After the clonogenic assay analysis, the slope of the survival curve by Do (the dose to reduce survival to 37% of its value at any point on the final near-exponential portion of the curve) was calculated. This dose (2 Gy for each cell line) was selected for further experiments.

### Immunofluorescence

2×105 cells/mL cells were cultured on 15 mm cover glasses. After 24 hours incubation, cells fixated in 4% paraformaldehyde (Sigma P-6148) for 30 minutes and were permeabilised with 0.25% Triton X-100 (Bio Basic Canada Inc.-C34H62O11) for 15 min and blocked with 1% bovine serum albumin (BSA Chem Cruz sc-2323) in 1X phosphate buffered saline (PBS). Primary antibodies, OPN (Proteintech 22952-1-AP),

MIF (Santa Cruz sc-271631), NFATc2 (Proteintech 22023-1-AP), p53 (Leica Biosystems NCL-p53-CM5p), mTOR (Bioss BS-3494R) and SSEA-1 (Santa Cruz sc-101462) were diluted 1/100 and incubated at +4oC overnight. Secondary antibodies (Invitrogen Alexa Fluor 488 A11034 anti rabbit, Invitrogen Alexa Fluor 555 A32727 anti mouse) were diluted 1/200 and incubated for an hour. Samples were mounted with Fluoroshield Mounting Medium with DAPI (Abcam ab104139). Samples were observed by the appropriate fluorescent filter by Olympus CellSens Entry (Japan) and analysed by five individuals independently in ImageJ which is public domain open-source software.

### Immunocytochemistry

Cells were cultured, fixed and permeabilised as the same way with immunocytochemistry procedure (above). Cells were treated with $H_2O_2$ (Merck Emprove exp.-K41544097)for 10 min and washed with PBS, blocked with 1% bovine serum albumin (BSA) in 1X phosphate buffered saline (PBS). Primary antibody p53 (Leica Biosystems) diluted at 1/100 ratio and cells incubated overnight in primary antibody. Biotinylated secondary antibody (ScyTek Laboratories SHP125) was diluted 1/200 and cells incubated in it for 40 min, then cells were treated with HRP streptomycin solution for 40 min and rinsed with PBS. DAB solution (ScyTek Laboratories ACK125) was applied for final colouring. Images were photographed by Avertv and analysed in ImageJ software. İmage J analysis made in 40× magnification. For every group min 100 cells were counted and evaluated by five different individuals.

### Cell Cycle

2×105 cells/mL cells were cultured on 15 mm cover glasses. After 24 hours incubation, cells were fixed with 70% ice-cold ethanol overnight and Cell cycle kit (Muse Millipore-2941162) instructions were followed.

### Statistical Analysis

To examine the association between radiation factors on cell cycle phase's results were evaluated by t-tests.

## RESULTS

### *Clonogenic Assay*

Control and radiated groups of GBM and Astrocytic cell lines were observed via Olympus BX50 (Japan) microscope for 12 days (Fig. 1) and at the end of the experiment, cell lines stained with crystal violet for quantitative analysis conducted by four independent expert individually (Fig. 2).

### *Immunofluorescence and Immunocytochemistry*

### SSEA-1

Control and experiment groups of GBMCs showed higher expression than ACs. All experiment groups shows higher expression of SSEA-1 on day 12 when compared to on day 1. Remarkably, GBMCs and ACs displayed increased expressions of SSEA-1 after radiotherapy treatment (Table 1) (Fig. 3).

### p53

It has been shown that p53, one of the basic proteins of the control points of the cell cycle, mediates transition from G1 phase to S phase and from G2 phase to mitotic division with other regulatory proteins in radiation-induced DNA damage [22]. Among tu-

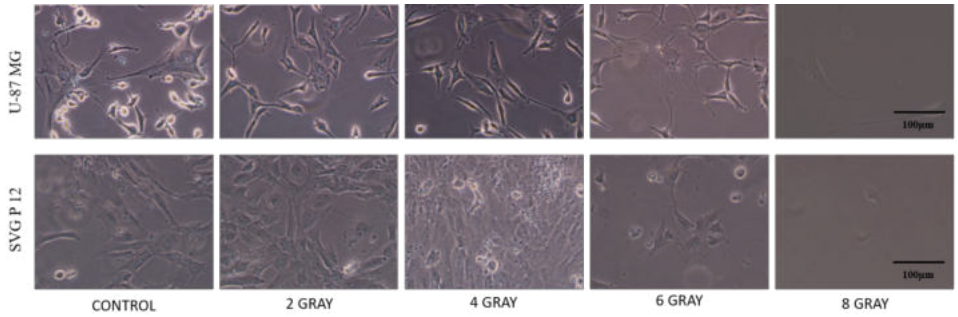


**Fig. 1. Phase-contrast images of GBMCs and ACs cells after 12 days of culturing of control and test groups. Images are at the same magnification (Scale bar 100 µm).**

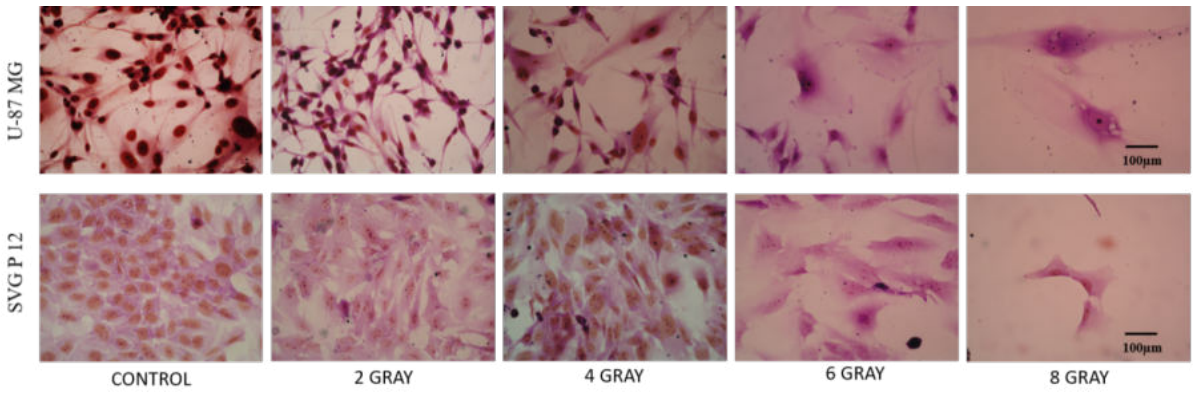


**Fig. 2. Colonogenic assay; GBMCs and ACs cells crystal violet staining. Cells were cultured for 12 days after radiotherapy, after fixation stained with crystal violet dye, and counted for control and test groups (Scale bar 100 µm)).**

**Table 1. SSEA-1 day 1 and day 12 IF results**

| | |
|---|---|
| **d1** | GBMC control < GBMC test |
| | AC control < AC test |
| | GBMC control > AC control |
| | GBMC test > AC test |
| **d12** | GBMC control < GBMC test |
| | AC control < AC test |
| | GBMC control > AC control |
| | GBMC test > AC test |
| **d1 vs d12** | GBMC control d12 > GBMC control d1 |
| | AC control d12 > AC control d1 |
| | GBMC test d12 > GBMC test d1 |
| | AC test d12 > AC test d1 |

SSEA-1 = stage-specific embryonic antigen-1, GBMC = glioblastoma multiforme cell, AC = astrocyte cell, d1 = day 1, d12 = days 12

mour suppressor genes, p53 plays an important role in the pathogenesis of many types of malignancies [13]. Radiation-sensitive cell lines exposed to 2 Gy radiation showed a significant increase in p53 within 8 hours [14]. According to our findings, on day 1 in GBMCs control group p53 expression was higher than GBMCs test group and on day 12 ACs test group p53 expression was higher than ACs control group. Both test groups of tumour cell line and astrocytic cell line showed increment in p53 expressions on day 12 which can indicate that in the long-term tissues or cells that suffering from ionized radiation effects, similarly up-regulates one of the key regulators of cell cycle and apoptosis (Table 2) (Fig. 4).

## mTOR

GBMCs control group's mTOR expression was higher than ACs control group, the result demonstrates

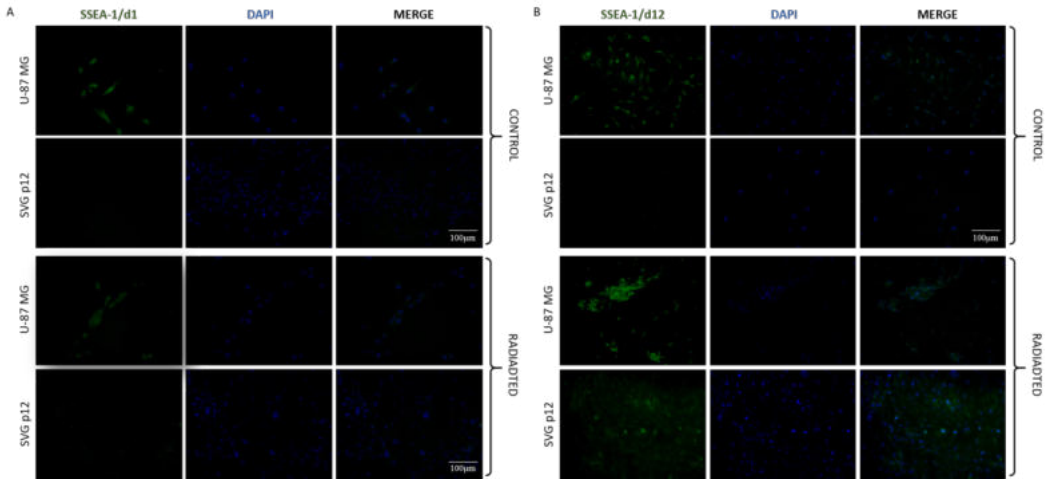


**Fig. 3. SSEA - 1 expressions of GBMCs and ACs cells after (A) 1 day and (B) 12 day of culturing (Scale bar 100 µm).**

**Table 2. p53 day 1 and day 12 IF results; The results for p 53 shows that GBMCs control group's p53 expression was higher than GBMCs experiment group on day 1**

| | |
|---|---|
| **d1** | GBMC control > GBMC test |
| | AC control < AC test |
| | GBMC Control ~ AC Control |
| | GBMC test < AC Test |
| **d12** | GBMC control < GBMC test |
| | AC control < AC test |
| | GBMC Control < AC Control |
| | GBMC test ~ AC Test |
| **d1 vs d12** | GBMC control d1 ~ GBMC control d12 |
| | AC control d1 ~ AC control d12 |
| | GBMC test d1 < GBMC test d12 |
| | AC test d1 < AC test d12 |

GBMC = glioblastoma multiforme cell, AC = astrocyte cell, d1 = day 1, d12 = days 12

that after radiotherapy treatment mTOR expressions are reduced (Table 3) (Fig. 5).

## MIF

After radiotherapy, ACs and GBMCs showed increased expression of MIF than control and after radiotherapy GBMCs showed higher expressional level than ACs (Table 4) (Fig. 6).

## OPN

According to our results in ACs after radiotherapy on day 1 low expression level observed in osteopontin and beside on day 12 test group ACs osteopontin high expression level observed compared to day 1 test group. On day 12 increasing of osteopontin expression observed in GBMCs test group compared to the GBMCs control group (Table 5) (Fig. 7).

## NFATc2

Increasing in NFATc2 expression in GBMCs after radiation treatment compared to GBMCs control group (Table 6) (Fig. 8).

### *Cell Cycle*

Both GBMCs and ACs control groups significantly accumulated in S phase. After radiotherapy application for GBMCs in G0/G1 and especially G2/M phase increasing and S phase decreasing observed. For ACs increasing S phase and decreasing in G0/G1 and G2/M phases observed (Fig. 9).

## DISCUSSION

Due to the resistance of traditional cancer treatment approaches, development of targeting therapies for TICs can be the destination of new approaches to the cancer treatments. To choose the key regulator targets, understanding the nature and the response to the external impacts of these cells are initial and inevitable. It is important to classify and isolate these cells from the tumour tissue by using surface markers such as SSEA-1. Collection for SSEA-1+ cells enriches for glioma tumour TIC subpopulations in all of the

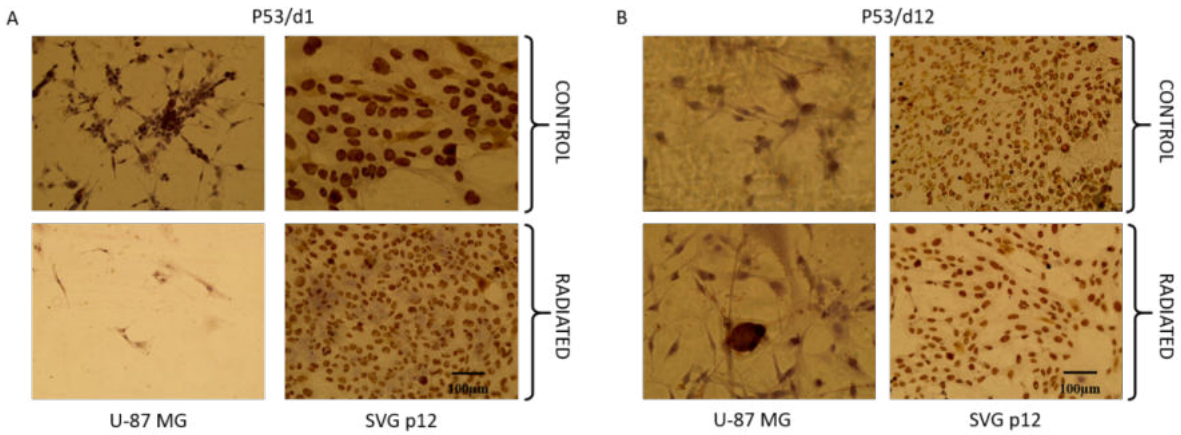


**Fig. 4. p53 expressions of GBMCs and ACs cells after (A) 1 day and (B) 12 day of culturing (Scale bar 100 µm).**

GBMCs [9].

In our experiments, we found that SSEA-1 expression of GBMCs both control and test group were higher than ACs control and test groups. However, every experimental groups presented increased expression of SSEA-1 on day 12 when compared with on day 1; in addition, after radiotherapy application, both GBMCs and ACs displayed increased expressions of SSEA-1 (Table 1) (Fig. 3). This might indicate that radiotherapy resistant and tumour initiating properties acquiring cells were survived.

**Table 3. mTOR day 1 IF results**

| d1 | GBMC control > GBMC test |
|---|---|
| | AC control ~ AC test |
| | GBMC control > AC control |
| | GBMC test > AC test |

mTOR = mammalian target of rapamycin, GBMC = glioblastoma multiforme cell, AC = astrocyte cell, d1 = day 1

For tumour cells present SSEA-1 show tumour initiating capacities more than mature astrocytic cells; however, with radiation stimulation both astrocytic and tumour cell line SSEA-1 expression increase observed. Radiotherapy application clearly is not sufficient for tumour therapy alone and afterwards resistant cells present more SSEA-1. Normal tissue is affected by the tumour cells because of the cancer cell microenvironment interaction. As a result of this interaction, SSEA-1 increase in both normal cells and tumour cells. Furthermore, this research present that the SSEA-1 increasing more significantly in the tumour cells, it might indicate the difficulty of treatment and the tumour relapses in vivo.

Studies have shown that DNA damage induced by ionizing radiation causes arrest in the G1 and G2 phases of the cycle in mammalian cells, and that this observation is related to radiation hypersensitivity [14]. Another finding is that transition from G2 phase to M phase is especially essential for provision of genomic stability and survival after ionizing radiation

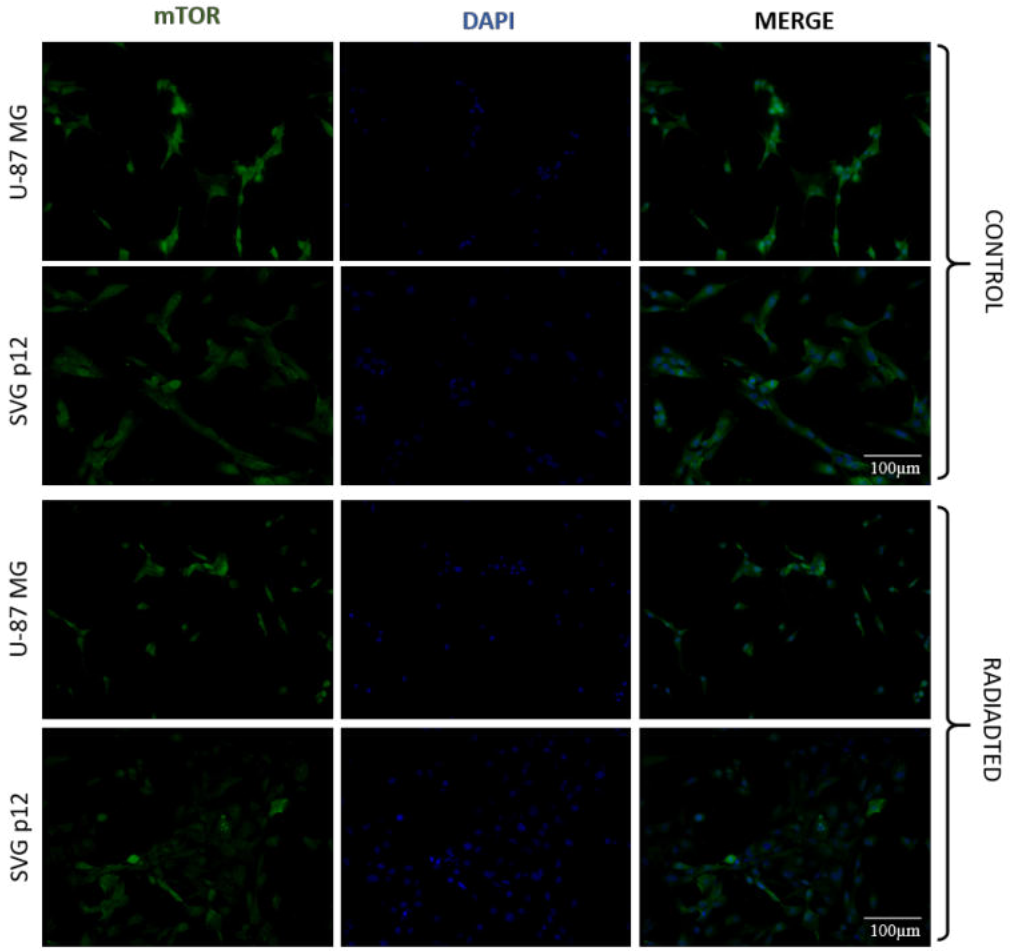


**Fig. 5. mTOR expressions of GBMCs and ACs cells after 1 day of culturing (Scale bar 100 µm).**

exposure [23].

Both GBMCs and ACs groups were significantly accumulated in S phase. After radiotherapy application for GBMCs in G0/G1 and especially G2/M phase increasing and S phase decreasing observed. These findings suggest that as a result to the radiotherapy effect, GBMCs started to accumulate on check points. On the

**Table 4. MIF day 1 IF results**

| | |
|---|---|
| **d1** | GBMC control < GBMC test |
| | AC control < AC test |
| | GBMC control > AC control |
| | GBMC test > AC test |

MIF = macrophage migration inhibiting factor, GBMC = glioblastoma multiforme cell, AC = astrocyte cell, d1 = day 1

**Table 5. OPN day 1 and day 12 IF results**

| | |
|---|---|
| **d1** | GBMC control ~ GBMC test |
| | AC control > AC test |
| | GBMC control ~ AC control |
| | GBMC test > AC test |
| **d12** | GBMC control < GBMC test |
| | AC control ~ AC test |
| | GBMC control ~ AC control |
| | GBMC test > AC test |
| **d1 vs d12** | GBMC control d12 < GBMC control d1 |
| | AC control 12 < AC control d1 |
| | GBMC test d12 < GBMC test d1 |
| | AC test d12 > AC test d1 |

OPN = osteopontin, GBMC = glioblastoma multiforme cell, AC = astrocyte cell, d1 = day 1, d12 = days 12

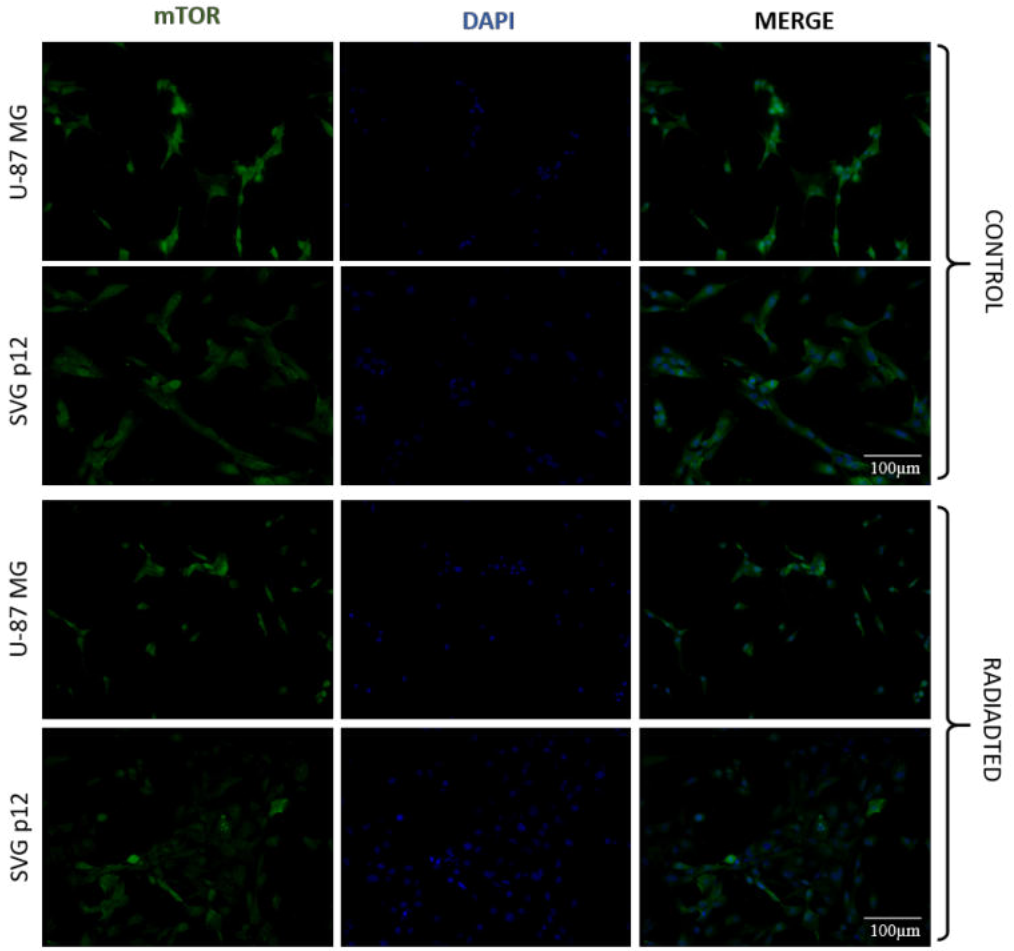


**Fig. 6. MIF expressions of GBMCs and ACs cells after 1 day of culturing (Scale bar 100 µm).**

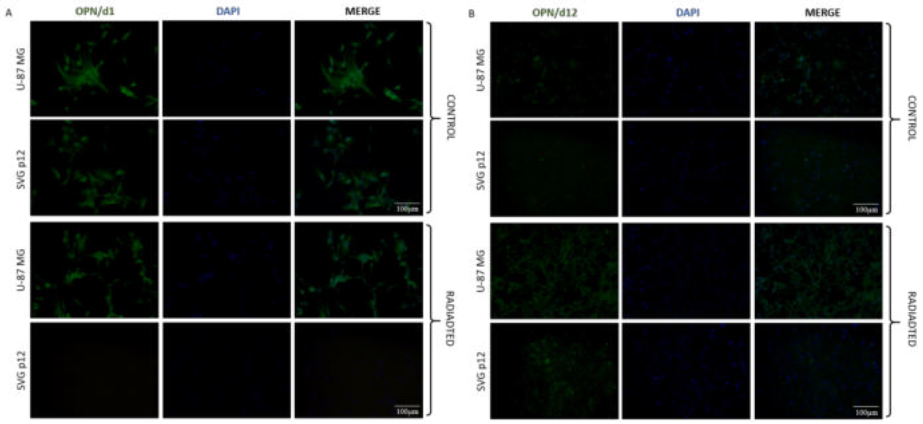


**Fig. 7.** **OPN expressions of GBMCs and ACs cells after (A) 1 day and (B) 12 day of culturing (Scale bar 100 µm).**

**Table 6.** **NFATc2 day 1 IF results**

| d1 | GBMC control < GBMC test |
|---|---|
| | AC control > AC test |
| | GBMC control ~ AC control |
| | GBMC test > AC test |

NFATc2 = nuclear factor of activated T-cells cytoplasmic 2, GBMC = glioblastoma multiforme cell, AC = astrocyte cell, d1 = day 1

other hand, ionized radiation affects normal tissue cells differently than tumour cells. For ACs increasing S phase and decreasing in G0/G1 and G2/M phases observed (Fig. 9).

Abnormal signalling in mTOR is may affect tumorigenesis and resistance treatment. p53 mutation, which is frequently seen in tumour formation, increases mTOR activation can be seen in glioblastomas that develops hyper activation of mTOR [24]. In a

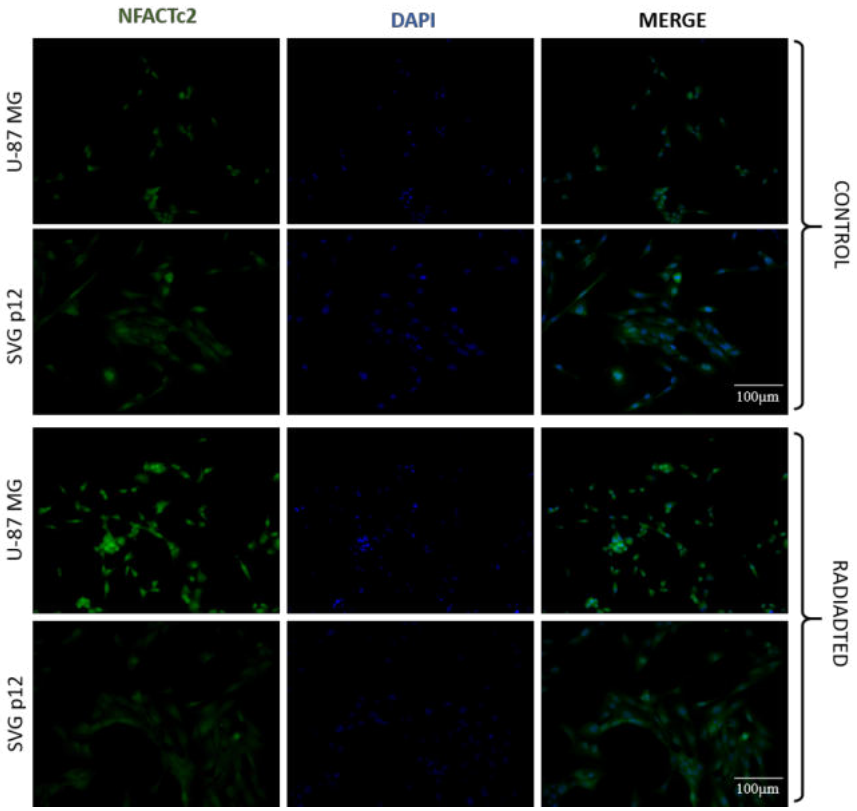


**Fig. 8.** **NFATc2 expressions of GBMCs and ACs cells after 1 day of culturing (Scale bar 100 µm).**

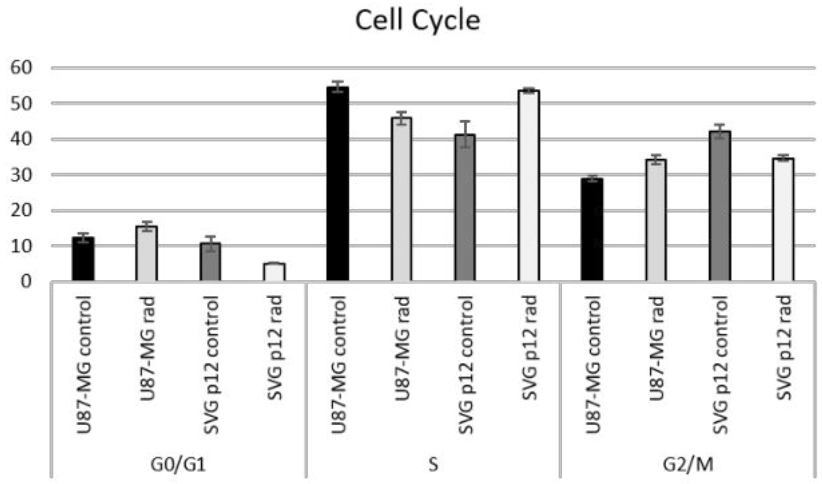


**Fig. 9. Cell cycle analysis of GBMCs and ACs cells after 1 day culturing.**

study, it was reported that mTOR inhibitors reduce the migration and invasion of GBMCs, also reduce the motility of these cells by the regulation of F-actin and paxillin [25]. In our experiments, mTOR activation was also found to be high in GBMCs with high p53 levels in accordance with the literature. At the same time, as a radiotherapy response, tumour cells and normal tissue cells responded similarly to the ionized radiation with decreasing in mTOR expressions in both GBMCs and ACs (Table 3) (Fig. 5). With treatment, reduced mTOR expressions may be supportive on consistency usage of the radiotherapy and mTOR inhibitors.

MIF expression is strongly associated with the mutational states and activity of p53 in GBMCs. A research study concluded that MIF is strongly expressed in astrocytomas and this increases with higher grades of malignancy [26]. As a cytokine, MIF is the indicator for angiogenesis, cell cycle and p53 which inducing apoptosis and effective in tumorigenesis [16]. Similarly, with the literature, results of this research show that MIF expressions higher in GBMCs more than ACs (Table 4) (Fig. 6). And via radiotherapy stimulation both tumour cell line and astrocytic cell line shows increment in MIF expressions. After radiotherapy tumour and astrocytic cell line increment in MIF expressions might be tone of the reasons for the difficulty of treating relapse tumours.

Researchers found that silencing of OPN expression in GBMCs leads to decrease cell migration and inhibits of tumour growth [27]. In another research stated that high OPN expression was associated with poor survival in GBM patients treated with radiotherapy. Also same researchers indicated that OPN depletion makes GBMCs more susceptible to radiation and DNA damage accumulation after irradiation is higher in these cells than in control cells [19]. In our experiments, we observed OPN expression in GBMCs both in day 1 and day 12. Even though GBMCs test group showed decreasing in OPN expressions on day 1, on day 12 test group OPN expression was higher as similar with ACs. After short time from ionized radiation application loss of OPN expression was found the ACs, however in the long term the OPN expression had increased (Table 5) (Fig. 7). The decrease on day 1 in GBMCs were indicating that loss of OPN expression worsening the effects of radiation the treatment response is compatible with the literature. However, increased OPN expressions, is in concordance with the increased SSEA-1 expressions on day 12. The surviving cells which are resistant and presenting stem cell like markers also displaying increased amount of OPN expressions. And in addition, regular tissue cells OPN pathway affected by the radiation.

According to our study, both GBMCs and ACs control group shows similar expressions of NFATc2 but via radiotherapy increasing in NFATc2 expression in GBMCs compared to GBMCs, ACs control and ACs test groups was found (Table 6) (Fig. 8). Radiotherapy may not affect normal tissue cells as the tumour cells when it comes to NFATc2 but the surviving tumour cells expressing more NFATc2 can contribute the invasiveness of the tumour cells.

## CONCLUSION

As a result, radiotherapy is a significant method for treatment of cancer and effects on cell signalling pathways are critical, especially in understanding cancer residues and recurrence. Tumour cells are not only the target of the therapeutics individually, but also cell-cell and cell extracellular matrix interactions act in tumour progression. Detecting cancer cells in the tissue and cells that may have tumour initiating capacities, and learning more about the intracellular and extracellular signal transduction of cells, are the key points that can lead to resolution of treatment failures. It should be kept in mind that when treatment is applied, not only cancerous tissue but also surrounding normal tissue cells will be affected and signal changes in these cells will be effective as tumour niche in both treatment success and tumour recurrences. Given molecular treatments, pathways that target tumour tissue but which will be least effective on the functions of normal tissue cells or cell fates, should be identified. After radiotherapy, it is important to show the change in the characteristics of the cells with time and to determine the tumour initiating properties of the surviving cells and treatment resistance. The determination of the cascade change of signal pathways after radiotherapy is indispensable in the target therapeutic model creation studies.

*Authors' Contribution*

Study Conception: DÇK, BÖ, YA; Study Design: DÇK, BÖ, YA, SH; Supervision: ESK, GÖ, AU, HA; Funding: ESK, GÖ, AU, HA; Materials: DÇK, BÖ, YA; Data Collection and/or Processing: BÖ, SH, ESK; Statistical Analysis and/or Data Interpretation: DÇK, BÖ, YA, SH, ESK; Literature Review: DÇK, BÖ, YA, HA; Manuscript Preparation: DÇK, BÖ, YA and Critical Review: GÖ, AU, HA.

*Conflict of interest*

The authors disclosed no conflict of interest during the preparation or publication of this manuscript.

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