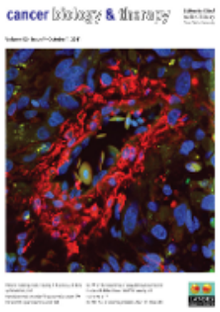

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RESEARCH PAPER

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Increased migration of a human glioma cell line after in vitro CyberKnife irradiation

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Keywords: glioma, radiation, clonogenic survival, invasion, CyberKnife

Abbreviations: GBM, glioblastoma multiforme; CK, CyberKnife; SRS, stereotactic radiosurgery; CIR, conventional irradiation; IIR, CK-driven intermittent irradiation

A human glioblastoma multiforme cell line (U87) and its derived-spheroids were irradiated either using a conventional irradiation (CIR) or a CK-like irradiation (IIR) in which the 8 Gy was delivered intermittently over a period of 40 min. The ability of glioma cells to migrate into a matrigel matrix was evaluated on days 1–8 from irradiation. Irradiation with CK-driven IIR significantly increased the invasion potential of U87 cells in a matrigel-based assay. In contrast to CIR, IIR was associated with increased levels of TGFβ at four days (real-time PCR), β1-integrin at 4–5 d (real-time PCR and protein gel blot) and no elevation in phosphorylated AKT at days 4 and 5 (protein gel blot). Our data suggests that glioma cell invasion as well as elevations of TGFβ and β1-integrin are associated with IIR and not CIR.

Introduction

Glioblastoma (GBM) is one of the most aggressive and lethal neoplasms of the brain. In spite of the multidisciplinary approach with surgical resection and radiotherapy with or without adjuvant chemotherapy,[1] the prognosis for patients with GBM remains poor.[2] The high prevalence of intrinsic or acquired radioresistance and the locally invasive growth of this tumor are two of the features that account for the high incidence of recurrences, even following maximal surgical resection.[3] Although multiple factors may be responsible for the ultimate outcome of GBM treatment, an increased emphasis on local tumor control has been recommended due to the fact that the majority of patients develop local recurrences, often within 2 cm of the initial tumor location.[4] Stereotactic radiosurgery (SRS) has been explored as an option for treating GBM patients especially in the context of glioblastoma relapse. Gamma Knife® and linear accelerator (LINAC)-based systems are the first two systems used for delivering SRS without surgical implantation of radiation sources.

The new vanguard type of SRS is the CyberKnife® (CK), a LINAC-based system used also for the treatment of patients with GBM. CK may be an alternative to conventional LINAC in the treatment of recurrent glioma with the advantage of reducing irradiation to normal tissue. Provided of an image-guided frameless radiosurgery system, CK treatment is delivered by a linear accelerator mounted on a flexible robotic arm. Since the total dose is delivered to the tumor usually through more than one hundred beams and some time is needed for the continuous alignment of the robotic arm to the target, in a typical CK treatment session the dose is released in a longer time compared with conventional LINAC irradiation (CIR).

In a recently published multicenter study, SRS with CK treatment was well tolerated although the median survival time did not exceed 7 mo (range, 1–34 mo) in patients with recurrent GBM.[5]

While the antiproliferative effects of irradiation on glioma cells have been extensively studied, little is known about the effects of ionizing radiation on migration/invasion of GBM cells and even less is known about potential differences in biological effects between different SRS irradiation modality. It has previously been reported that in vitro cultured GBM cells may increase their migration/invasion ability after irradiation.[6] However, other authors did not report differences after irradiation with various schedules.[7]

It has been recently shown that blocking of β1 integrin significantly impaired the adhesion and consequent migration of glioma cells;[8] as a matter of fact, the adhesion of tumor cells to the extracellular matrix is mediated by β1 integrin that was believed to link the growing actin filaments at the leading edge to the substratum and to initiate or coordinate signals.[9] Other pathways, such as the PI3K/Akt have been described to be implicated in

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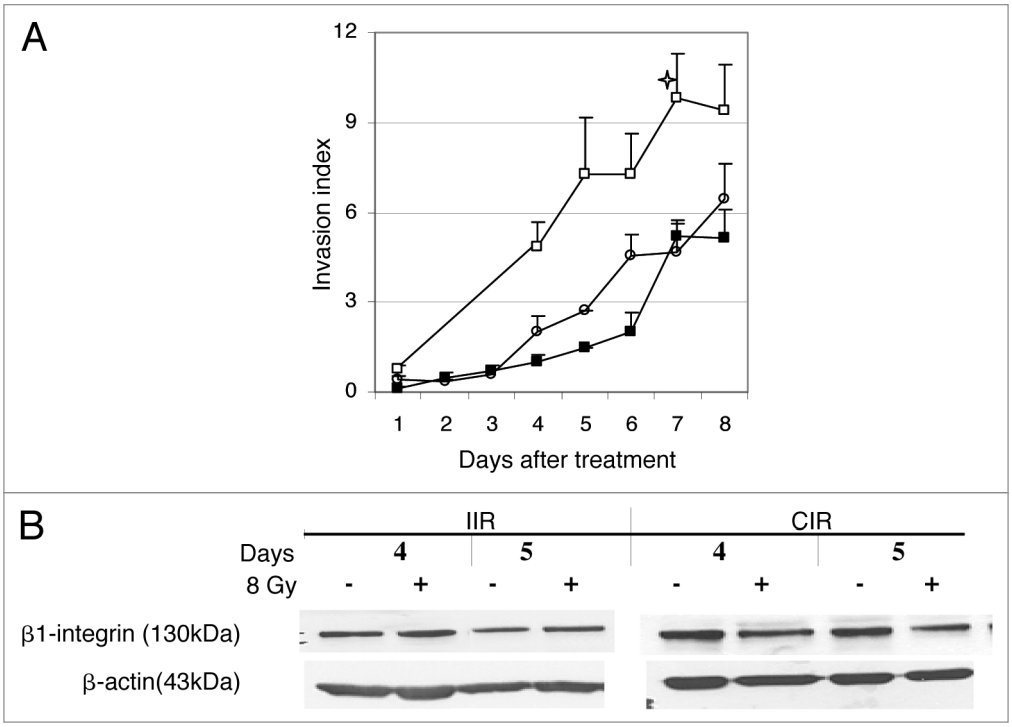

Figure 1. Invasion indexes of U87. Invasion indexes of U87 calculated as described in the methods section (mean ± SE). (A) Data were obtained at different days after treatment with 8 Gy (control-○, CIR-■ and IIR-□). A significant increase in invasion ability was detected after IIR treatment ($p = 0.032$; Mann Whitney test), whereas no significant differences were detected after CIR treatment. All results are means of at least three indipendent experiments. (B) β1-integrin expression in U87 spheroids. Spheroids of U87 were exposed to 8 Gy (CIR or CK-driven IIR) and cultured in Matrigel for the following days. Treatment with CK-driven IIR induced an increase in β1-integrin expression, as detected by protein gel blot analysis.

migration/invasion, although the signaling events regulating cell motility are poorly defined.[10]

In the present study we analyzed the in vitro effects of irradiation on the migration of glioma cells, using a three dimensional model. Spheroids of glioma cells were treated either by CIR or by CK-driven intermittent irradiation (IIR) and migration of glioma cells from the spheroids invading the matrix was assessed for the following days. The results suggest that, in our experimental model, CK-IIR increase migration of glioma cells through the upregulation of β1-integrin expression.

Results

Treatment with CK-driven IIR (8 Gy) significantly increased the migration ability of spheroid-derived glioblastoma cells, compared with untreated spheroids (**Fig. 1A**). Despite this effect was already detectable after 4 d from irradiation, the difference from the control was statistically significant after 7 d ($p = 0.032$). Treatment with CIR did not induce any significant change in invasion ability compared with control. Western blot analysis showed that β1-integrin was downregulated in glioblastoma cells after treatment with CIR at all time tested, while the opposite was found after exposure to CK-driven IIR (**Fig. 1B**).

To further investigate these findings, real-time PCR analysis was performed on U87 spheroids at the same time points (**Fig. 2**). Indeed, a 3-fold increase in β1-integrin transcripts was detectable in spheroids treated with CK-driven IIR at the fourth day of the invasion assay. Moreover, in IIR-treated spheroids, a 5-fold increase in TGFβ was also noticed while no significant variations in β1-integrin or TGFβ transcription were found after treatment with CIR (**Fig. 2**).

To detect a possible involvement of the anti-apoptotic Akt pathway in migration,[10] total and phophorytated Akt were also analyzed by protein gel blot. The Akt pathway was activated in spheroids treated with CIR since phosphorylated-Akt was increased after a total dose of 8 Gy while no significant differences were found in total Akt levels (**Fig. 3**). On the other hand, although a moderate increase in total Akt was detected in cells treated with CK-driven IIR, a slight decrease in phosphoryted-Akt was detected suggesting that this survival pathway was not upregulated by this irradiation modality.

Discussion

Malignant gliomas are pathophysiologically characterized by their insidious infiltration of the brain.[11] Within the conventional LINAC treatment for brain tumors, the new vanguard type of stereotactic radiotherapy, the CyberKnife, is becoming more frequently used for the treatment of recurrences. Still, in vitro data about the effects of CyberKnife on glioma cells are lacking in literature, therefore a comparison of radiation-induced invasion of glioma cells using these two treatment modalities seems reasonable.

In our experimental conditions, glioma cells treated with CK could be considered as submitted to a fractionation schedule composed to 161 fraction of 4.6 ± 3.4 cGy (median ± SD) each every 10.9 ± 0.51 sec (median ± SD).

Our data showed that, at a total dose of 8 Gy, in vitro treatment with CK-driven IIR resulted in a significantly increased invasion of glioma cells; moreover our data suggest that β1-integrin is a key regulator in invasion of these cells. In fact, in our experimental model, a direct relation between migration of glioma cells and β1-integrin expression was detected.

Even if both treatments decreased significantly the survival of U87 (data not shown), the invasion potential tends to increase after CK-driven IIR and decrease after CIR irradiation. As a matter of fact, β1 integrin has been implicated in mediating resistance to IR;[12] in a breast cancer model, the combination of anti β1-integrin antibody and IR has been reported to maximize the effects of the irradiation, suggesting that β1 integrin is a key regulator of post-irradiation tumor cell survival.[13]

Our observations are in agreement with a previous work showing enhanced migration and invasion of irradiated glioma cells 24 h after irradiation.[6] On the other hand, in a previously published work where spheroids from the same glioma cell lines (U87) were irradiated with two types of fractionated radiotherapy, no differences in migration were detected nor if compared with untreated cells nor between the two irradiation modality.[7] However, for the invasion studies they used a co-culture system in which tumor spheroids were confronted with fetal rat brain aggregates, therefore differences in the experimental model might explain the conflicting results. Indeed, β1-integrin upregulation has been described to occur after clinically relevant doses of ionizing radiation in melanoma and lung cancer cell lines, but the type of matrix used has been shown to have a role in β1-integrin modulation, being fibronectin and laminin the most effective matrices.[13,14] Since the major components of Matrigel is laminin, our experimental conditions possibly maximize differences between irradiation modality.

Moreover, we showed that the modulation of β1-integrin expression was secondary to an increase in TGFβ as detected by realtime PCR. This was not surprising since upregulation of TGFβ after multifraction irradiation in gliomas has been previously reported in reference 15, due to the well known immunosuppressive properties of TGFβ, therefore this effect of IIR on glioma cells may be undesirable. Moreover, TGFβ has been shown to enhance motility and invasiveness of oral squamous carcinoma cell lines via modulation of a number of integrins, including β1-integrin.[16] Our data suggest that the same mechanism might be activated in glioma cell lines.

In contrast with previously reported data, in our migration model the Akt pathway did not correlate with the migration ability.[10] In fact phospho-Akt was detectable after treatment with CIR, while no activation was detectable in cells treated with CK-driven IIR. The fact that CIR and CK-driven IIR differs in the activation of a pathway involved also in cell survival, suggests that the two irradiation modality might differ also in cell killing efficiency.

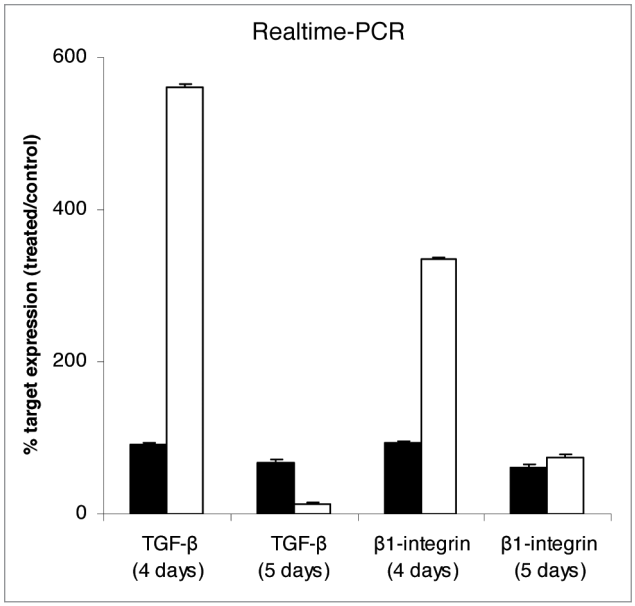

Figure 2. Expression levels of TGFβ and β1-integrin in U87. Expression levels of TGFβ and β1-integrin genes as detected by realtime-PCR in U87 spheroids 4 and 5 d after irradiation. GAPDH was used as housekeeping gene. Data are expressed as mean percentage variation of target expression from untreated cells at different time points (100% means no modulation). ■: CIR (conventional LINAC irradiation); □: IIR (CK-driven intermittent irradiation).

The central role of β1-integrin in cancer cell migration has been recently demonstrated by the identification of SCAI, a novel highly conserved protein that regulates β1-integrin transcription. Depletion of SCAI caused a drastic upregulation of β1-integrin gene expression levels which resulted in a strong increase in migration in three-dimensional matrices of different cancer cell types.[17] Further study are needed to verify a role for SCAI in radiation-induced β1-integrin upregulation in glioma.

Material and Methods

Spheroid culture. Spheroids were obtained from U87 cells (ATCC, American Type Culture Collection) cultured in complete medium (Dulbecco's Modified Eagle's supplemented with 10% fetal bovine serum and 0.01% penicilline/streptamicine) at 37°C in humidified atmosphere of 5% CO_2 in air. 2.5 x 10^5 cells were seeded on an agarose (Biorad) layer in a 60 x 15 mm Petri and left for 2 d in incubator for spheroids formation with 3 ml of complete medium.

Irradiation. Cells cultured as monolayer or spheroids were irradiated in culture dish at room temperature. Conventional LINAC irradiation (CIR) was performed with a 6 MV X-rays uniform beam produced by a Philips SL 75/5 (Elekta) linear accelerator with a dose/rate of 2 Gy/min for field sizes of 20 cm x 20 cm, gantry = 180° and source-to-surface distance was fixed at 100 cm. The water-solid RW3 were used to assure the 100% of the dose in the build-up region on cells.

The CK-driven irradiation (IIR) (Accuray Inc.) was performed delivering a total dose of 8 Gy with a dose rate of 4 Gy/min. The

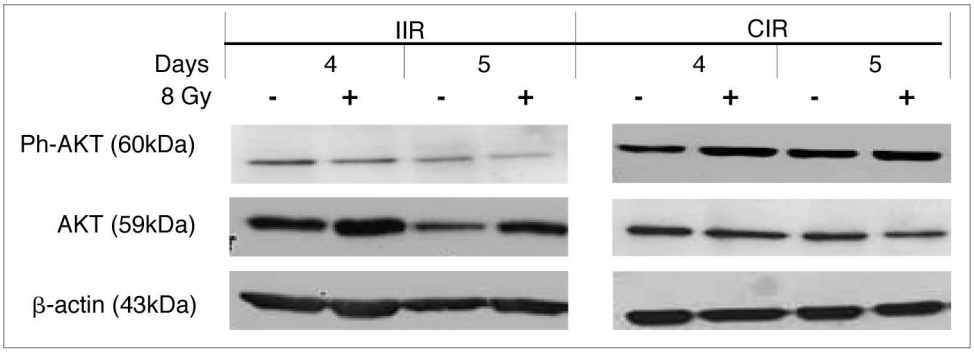

Figure 3. Radiation-induced activation of the AKT pathway in U87 spheroids. Spheroids of U87 were exposed to 8 Gy (CIR or CK-driven IIR), cultured in Matrigel for the following days and harvested at different time-points. Protein gel blot analysis showed that after treatment with CIR the expression of phospho-Akt was upregulated while no differences were found in total Akt levels. On the other hand, treatment with CK-driven IIR did not modify phospho-AKT levels although a slight increase in total AKT was observed.

plan utilized a 60 mm diameter collimator that delivered anywhere from 0.30–17.96 cGy (median ± standard deviations (SD) = 4.6 ± 3.4 cGy) per beam from 161 distinct directions with a median interval between two beams of 10.9 ± 0.51 sec. The prescription line isodose was 80% and the irradiation duration was about 40 min. The cells, in Petri dishes, were put in a human-head-like phantom, so that the CK was able to align the target and perform the treatment.

Cells and spheroids not undergoing radiation treatment, were left at room temperature for the same period of time as controls.

Matrigel invasion assay. After exposure to 8 Gy, with CIR or CK-driven IIR, single spheroids with a maximum diameter of 200 μm (checked by an inverted microscope equipped with a eye-piece graticule) were pipetted and transferred into ice-cold 96-multiwell plate wells (one spheroid/well) previously filled with 100 μl Matrigel® (BD Bioscience) diluted 1:3 with serum-free medium. Plates were then left at 37°C for 30 min to allow Matrigel polymerization before 200 μl complete medium was added to each well.

Maximal cell migration (D) was determined daily for the following 10 d using an inverted microscope equipped with a eye-piece graticule calibrated against a micrometer slide. The invasion area was calculated by the following formula: $\pi \cdot (D/2)^2$ in mm^2 and the initial spheroid area subtracted.[18] At least five spheroids for each treatment group for each experiment were transferred.

Protein gel blot analysis. At different days after irradiation, spheroids left in matrigel were collected and homogenized in lysis buffer containing 62.5 mM TRIS-HCl pH 6.8, 10% glycerol, 2% SDS, 5% β-mercaptoethanol. Protein concentration was determined by Quant-iT protein fluorometric assay for QBIT fluorometer (Invitrogen-Molecular Probes).

Spheroids lysates were separated in a 10% SDS-polyacrylamide gel and then transferred to a Hybond-P PVDF membrane (GE Healthcare) at 350 mA for 1 h. Thereafter, the membranes were blocked for one hour at room temperature with 5% non fat dry milk in TPBS (1x phosphate-buffered saline, 0.1% Tween 20), then incubated with 1 μg/ml of the appropriate antibody, anti-Akt mouse IgG (Transduction Laboratories), anti-phopsho-Akt (Ser 473) rabbit IgG and anti-β1-integrin rabbit policlonal IgG (Chemicon), overnight at 4°C. Following two washes with TPBS, membranes were incubated for one hour at room temperature with 1:2,500-diluted horseradish-peroxidase-conjugated goat anti-mouse or anti-rabbit immunoglobulins (DAKO) respectively. PVDF membranes were washed three times with TPBS then evenly coated using the ECL protein gel blotting detection system (GE Healthcare) for 1 min. Membranes were immediately exposed to AGFA Curix Ortho X-Ray film (AGFA). To asses equal protein loading onto gel, membranes were reprobed, after removal of previous antibodies, with mouse anti-β actin IgG (SIGMA).

Realtime PCR. Spheroids of U87 were exposed to 8 Gy (CIR and CK-driven IIR) and cultured in Matrigel for the following days. Total RNA was isolated from spheroids using a commercially available kit following the manufacturer's instructions (Qiagen). Total RNA from each sample was reverse-transcribed by random priming and Moloney murine leukemia virus reverse transcriptase (Promega). Real time PCR was performed with commercially available reagents in an SDS-5700 instrument following the manufacturer's protocol (Applied Biosystems). One hundred nanograms of retro-transcribed RNA was amplified for β1-integrin, TGFβ and Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as control. All reactions were performed in duplicate and the mean Ct (cycle threshold) value was used as index of expression.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

References

1. Stupp R, Dietrich PY, Ostermann Kraljevic S, Pica A, Maillard I, Maeder P, et al. Promising survival for patients with newly diagnosed glioblastoma multiforme treated with concomitant radiation plus temozolomide followed by adjuvant temozolomide. J Clin Oncol 2002; 20:1375-82; PMID:11870182; DOI:10.1200/JCO.20.5.1375.
2. Galanis E, Buckner J. Chemotherapy for high-grade gliomas. Br J Cancer 2000; 82:1371-80; PMID:10780513.
3. Giese A. Glioma invasion-pattern of dissemination by mechanisms of invasion and surgical intervention, pattern of gene expression and its regulatory control by tumorsuppressor p53 and proto-oncogene ETS-1. Acta Neurochir Suppl 2003; 88:153-62; PMID:14531573.
4. Masciopinto JE, Levin AB, Mehta MP, Rhode BS. Stereotactic radiosurgery for glioblastoma: a final report of 31 patients. J Neurosurg 1995; 82:530-5; PMID:7897511; DOI:10.3171/jns.1995.82.4.0530.
5. Villavicencio AT, Burneikiene S, Romanelli P, Fariselli L, McNeely L, Lipani JD, et al. Survival following stereotactic radiosurgery for newly diagnosed and recurrent glioblastoma multiforme: a multicenter experience. Neurosurg Rev 2009; 32:417-24; PMID:19633875; DOI:10.1007/s10143-009-0212-6.
6. Wild-Bode C, Weller M, Rimner A, Dichgans J, Wick W. Sublethal irradiation promotes migration and invasiveness of glioma cells: implications for radiotherapy of human glioblastoma. Cancer Res 2001; 61:2744-50; PMID:11289157.
7. Gliemroth J, Feyerabend T, Gerlach C, Arnold H, Terzis AJ. Proliferation, migration and invasion of human glioma cells exposed to fractionated radiotherapy in vitro. Neurosurg Rev 2003; 26:198-205; PMID:12845549.
8. Zhang B, Gu F, She C, Guo H, Li W, Niu R, et al. Reduction of Akt2 inhibits migration and invasion of glioma cells. Int J Cancer 2009; 125:585-95; PMID:19330838; DOI:10.1002/ijc.24314.
9. Webb DJ, Parsons JT, Horwitz AF. Adhesion assembly, disassembly and turnover in migrating cells—over and over and over again. Nat Cell Biol 2002; 4:97-100; PMID:11944043; DOI:10.1038/ncb0402-e97.
10. Zhou GL, Zhuo Y, King CC, Fryer BH, Bokoch GM, Field J. Akt Phosphorylation of Serine 21 on Pak1 Modulates Nck Binding and Cell Migration. Mol Cell Biol 2003; 23:8058-69; PMID:14585966; DOI:10.1128/MCB.23.22.8058-69.2003.
11. Kleihues P, Cavenee WK. Pathology and genetics: tumors of the central nervous system. Lyon, FR: IARC Press 2000.
12. Cordes N, Seidler J, Durzok R, Geinitz H, Brakebusch C. Beta1-integrin-mediated signaling essentially contributes to cell survival after radiation-induced genotoxic injury. Oncogene 2006; 25:1378-90; PMID:16247454; DOI:10.1038/sj.onc.1209164.
13. Park CC, Zhang HJ, Yao ES, Park CJ, Bissell MJ. Beta1 integrin inhibition dramatically enhances radiotherapy efficacy in human breast cancer xenografts. Cancer Res 2008; 68:4398-405; PMID:18519702; DOI:10.1158/0008-5472.CAN-07-6390.
14. Cordes N, Blaese MA, Meineke V, Van Beuningen D. Ionizing radiation induces upregulation of functional β1-integrin in human lung tumour cell lines in vitro. Int J Radiat Biol 2002; 78:347-57; PMID:12020426; DOI:10.1080/09553000110117340.
15. Tsai MH, Cook JA, Chandramouli GV, DeGraff W, Yan H, Zhao S, et al. Gene expression profiling of breast, prostate and glioma cells following single versus fractionated doses of radiation. Cancer Res 2007; 67:3845-52; PMID:17440099; DOI:10.1158/0008-5472.CAN-06-4250.
16. Takayama S, Hatori M, Kurihara Y, Kinugasa Y, Shirota T, Shintani S. Inhibition of TGFbeta1 suppresses motility and invasiveness of oral squamous cell carcinoma cell lines via modulation of integrins and downregulation of matrix-metalloproteinases. Oncol Rep 2009; 21:205-10; PMID:19082463.
17. Brandt DT, Baarlink C, Kitzing TM, Kremmer E, Ivaska J, Nollau P, et al. SCAI acts as a suppressor of cancer cell invasion through the transcriptional control of beta1-integrin. Nat Cell Biol 2009; 11:557-68; PMID:19350017; DOI:10.1038/ncb1862.
18. Bauman GS, Fisher BJ, McDonald W, Amberger VR, Moore E, Del Maestro RF. Effects of radiation on a three-dimensional model of malignant glioma invasion. Int J Dev Neurosci 1999; 17:643-51; PMID:10571424; DOI:10.1016/S0736-5748(99)00023-4.