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Original research article

Effects of γ-radiation on cell growth, cell cycle and promoter methylation of 22 cell cycle genes in the 1321NI astrocytoma cell line

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ABSTRACT

Purpose: DNA damage caused by radiation initiates biological responses affecting cell fate. DNA methylation regulates gene expression and modulates DNA damage pathways. Alterations in the methylation profiles of cell cycle regulating genes may control cell response to radiation. In this study we investigated the effect of ionizing radiation on the methylation levels of 22 cell cycle regulating genes in correlation with gene expression in 1321NI astrocytoma cell line.
Methods: 1321NI cells were irradiated with 2, 5 or 10 Gy doses then analyzed after 24, 48 and 72 h for cell viability using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliu bromide) assay. Flow cytometry were used to study the effect of 10 Gy irradiation on cell cycle. EpiTect Methyl II PCR Array was used to identify differentially methylated genes in irradiated cells. Changes in gene expression was determined by qPCR. Azacytidine treatment was used to determine whether DNA methylation affectes gene expression.
Results: Our results showed that irradiation decreased cell viability and caused cell cycle arrest at G2/M. Out of 22 genes tested, only *CCNF* and *RAD9A* showed some increase in DNA methylation (3.59% and 3.62%, respectively) after 10 Gy irradiation, and this increase coincided with downregulation of both genes (by 4 and 2 fold, respectively).
Treatment: with azacytidine confirmed that expression of *CCNF* and *RAD9A* genes was regulated by methylation.
Conclusions: 1321NI cell line is highly radioresistant and that irradiation of these cells with a 10 Gy dose increases DNA methylation of *CCNF* and *RAD9A* genes. This dose down-regulates these genes, favoring G2/M arrest.

1. Introduction

Astrocytoma is a malignant glioma which is one of the most devastating and lethal forms of human cancers. This is due to the highly penetrating nature of astrocytomas into normal adjacent brain tissue, and their relative radio- and chemoresistant character [1]. Patients diagnosed with astrocytoma die within a period of time ranging from months to a few years, and this has not significantly changed in the last 20 years despite the use of advanced surgery, radiation, and chemotherapy [2]. Gliomas patients are routinely treated with radiotherapy despite the radioresistance of this refractory tumor [3,4]. Ionizing radiation induces DNA damage, especially double strand breaks (DSB) that can significantly influence cell fate [5,6]. After DNA damage occurs, DNA damage pathways are activated, leading to cell cycle arrest. Following this arrest, cells can either repair the damage, proliferate, differentiate, become senescent, or enter apoptosis [6,7]. These pathways could be influenced by modulation of gene expression through epigenetic mechanisms, such as DNA methylation, miRNAs, or histone modifications [8,9]. DNA methylation is the most characterized epigenetic mechanism that occurs at CpG dinucleotides and influences many cellular processes by regulating gene transcription [8,10]. DNA is methylated through enzymes called DNA methyltransferases (DNMTs) [11]. Generally, hypermethylation of gene promoters is associated with reduced transcription, while hypomethylation increases gene expression [9]. Methylation patterns are frequently altered in cancer cells, with global DNA hypomethylation, and hypermethylation at specific CpG islands [12,13]. Hypermethylation may lead to silencing of tumor suppressor genes and hypomethylation can

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reactivate oncogenes [14,15]. Aberrant DNA methylation can also be induced in cells through exposure to chemicals or radiation [16–21]. Although recent studies showed that ionizing radiation causes global DNA hypomethylation [21–23], there is little information on changes in DNA methylation induced by radiation at specific loci. Previous studies showed that altering the methylation profile of cell cycle genes can modulates the response of cancer cells to radiation and determines their radiosensitivity [24,25]. Previous research demonstrated that promoter methylation of *ATM* (involved in a cell cycle checkpoint) and *ERCC1* (involved in DNA repair) correlated with radiosensitivity in several human glioma cell lines [26,27].

In view of the importance of understanding mechanisms of resistance of malignant gliomas to radiotherapy, we chose the 1321NI human astrocytoma cancer cell line as model to investigate the effects of irradiation on cell survival and on key genes controlling the cell cycle. The identification of specific genes as targets for radiosensitizing for combination treatment of the disease could improve outcome of radiotherapy in the future.

2. Methods

2.1. Cell culture

The human 1321NI cell line was purchased from Health Protection Agency Culture Collections (Salisbury, UK). Cells were maintained in DMEM (Gibco, USA) containing 10% fetal bovine serum (Gibco, USA), 2 mM L-Glutamine (Gibco, USA), 1% penicillin-streptomycin (Gibco, USA) at 37 °C in a 5% CO_2 humidified atmosphere (Binder, USA).

2.2. Cell irradiation

Cells were irradiated at room temperature with a Co^{60} gamma ray irradiator (Theratron 80, Springfield, Virginia, USA) at a dose rate of 447 mGy per minute for a total dose of 2, 5, or 10 Gy. Control cells were sham irradiated and manipulated in parallel with the tested cells.

2.3. MTT assay

Cells from a stock cultures were plated into 96-well-plates (5×10^3 cells/well, 100 μl medium) for MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay and allowed to attach overnight. Irradiation with 2, 5, and 10 Gy was performed on the next day. 10 μl MTT solution (Roche, Germany) was added to each well and incubated for 4 h at 37 °C. Then, 100 μl DMSO were added to each well, and absorbance of converted MTT was measured at 450 nm (reference at 690 nm) using Multiskan Ascent absorbance plate reader (Thermo Labsystems, Germany). The survival curve and SF-values (survival fraction) were determined as previously described [28]. We also measured Cell viability after 24, 48, and 72 h of radiation treatment as following:

Cell viability (%) = (average OD value of experimental group/average OD value of control group)*100%

2.4. Flow cytometric analysis of the cell cycle

1321NI cells (1×10^5 cells/ml) were treated with a 10 Gy dose of ionizing radiation and returned to the incubator for 24 h. Cells were collected, washed with PBS and fixed in 70% ethanol and incubated with propidium iodide (PI, BD Biosciences, USA) and 1 mg RNase in the dark for 30 min at 37 °C. For each sample, 1×10^4 cells were analyzed using the BD FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA) to determine the percentages of cells in each phase of the cell cycle.

2.5. DNA extraction

Genomic DNA from cultured 1321NI cells was extracted using the QIAamp DNA Mini kit (Qiagen, Germany) according to the manufacturer recommendations. DNA was quantified spectrophotometrically using NanoVue Plus (Bioch Rom, UK).

2.6. EpiTect methyl qPCR array analysis

Methylation status of 22 cell cycle genes were analyzed using EpiTect Methyl II PCR Array Human Cell Cycle, Signature Panel (EAHS-201ZC-2, Qiagen, Germany). This array profiles the promoter methylation status of a panel of 22 genes [G1 Phase CCND1, CCNE1, CDK4, CDKN1*B* (*p27KIP1*) – S Phase & DNA Replication: *MCM2*, *MCM4*- G2 Phase & G2/M Transition: *CCNB1*, *CDK5RAP1*, *CKS1B* – M Phase: *CCNF*, *MRE11A*, *RAD51*- Cell Cycle Checkpoint & Cell Cycle Arrest: *ATM*, *BRCA1*, *BRCA2*, *CDK2*, *CDKN1A* (*p21CIP1/WAF1*), *CDKN1B* (*p27KIP1*), *CHEK1*, *GADD45A*, *RAD9A*, *TP53*- Regulation of Cell Cycle: *ATM*, *BRCA1*, *BRCA2*, *CCNB1*, *CCND1*, *CCNE1*, *CCNF*, *CDK2*, *CDK4*, *CDKN1A* (*p21CIP1/WAF1*), *CDKN1B* (*p27KIP1*), *CKS1B*, *GADD45A*, *RAD9A*, *RBL1*, *TP53*]. The method of EpiTect Methyl II PCR array to determine methylation status is based on the detection of remaining input DNA by real-time PCR after digestion with methylation-sensitive (Ms) and methylation-dependent (Md) restriction enzymes. Genomic DNA from 10 Gy irradiated and non-irradiated 1321NI cells was digested using EpiTect Methyl II DNA Restriction Kit (Qiagen, Germany) according to the manufacturer's instructions and as describe below; a reaction mix without enzymes was included 1 μg genomic DNA, 26 μl of 5 × Restriction Digestion Buffer, and RNase-DNase free water was added to a final volume of 120 μl. Four digestion reactions (Mo, Ms, Md, and Msd) were prepared. Each one contained 28 μl of the previous reaction mix and 2 μl of RNase-DNase free water for Mo digest, 1 μl of methylation sensitive enzyme A and 1 μl of RNase-DNase free water for Ms digest, 1 μl methylation sensitive enzyme B and 1 μl of RNase-DNase free water for Md digest, 1 μl of methylation sensitive enzyme A and 1 μl of methylation sensitive enzyme B for Msd digest. All 4 tubes were incubated for 6 h at 37 °C. Then Real-time PCR was performed using RT2 SYBR Green ROX qPCR Master mix (Qiagen, Germany) according to the manufacturer's recommendations in a Step One plus Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Obtained threshold cycle (Ct) values were used to calculate the percentages of methylated (M) and unmethylated (UM) DNA, using a quantization algorithm provided by the manufacturer (Qiagen, Germany).

2.7. Azacytidine treatment

5-Azacytidine (Sigma-Aldrich, USA) was dissolved in DMSO to a concentration of 0.25 mM and passed through a sterile μStar plastic syringe filter (Non pyrogenic filter, USA) and aliquoted. Cells were grown in 5 ml DMEM overnight and 20 μl of azacytidine solution were added, then cells were returned to the incubator for 24 h. Addition of 20 μl azacytidine and incubation for 24 h were repeated twice (72 h total incubation).

2.8. Gene expression analysis by real-time PCR

Total RNA was extracted from azacytidine treated and non-treated 1321NI cells, and from 10 Gy irradiated and non-irradiated cells using the RNeasy Mini kit (Qiagen, Germany). Complementary DNA (cDNA) was synthesized from 3 μg RNA using M-MLV reverse transcriptase (Invitrogen, USA) for 1 h at 37 °C following

the manufacturer's instructions. Real-Time PCR was performed using a Step One plus cycler (Applied Biosystems, USA). To calculate the relative expression of *CCNF* (*Cyclin F*), *RAD9A* (*RAD9 homolog A*), *CCNE1* (*Cyclin E1*) genes, quantitative real-time PCR was performed using SYBR Green Supermix (BIO-RAD, USA) for 40 amplification cycles. Relative transcript quantities were calculated using the ΔΔCt method with *GAPDH* as reference gene. All reactions were run in triplicate. Primer sequences are detailed in Table 1.

2.9. Statistical analysis

The SPSS 22 software system (SPSS, Chicago, IL) was used for statistical analysis. The differences between experimental groups were analyzed using the Student-t test. Differences were considered statistically significant at $P < 0.05$.

3. Results

3.1. Effect of ionizing radiation on 1321NI cell survival

The initial effect of irradiation on 1321NI cell survival was measured by the MTT assay. A statistically significant dose-dependent decrease in surviving fraction (SF) was observed with increasing doses of radiation. As shown in Fig. 1, the SF decreased from 0.79 to 0.69 and to 0.58 after 2 Gy, 5 Gy and 10 Gy exposure, respectively. Since the observed SF2 (0.79) of the 1321NI cell line was greater than 0.5, we concluded that this cell line is highly radioresistant.

3.2. Effect of ionizing radiation on cell viability

As shown in Fig. 2, treating 1321NI cells with single doses of ionizing radiation (2, 5, and 10 Gy) caused a dose-dependent decrease in cell proliferation. We found that cell proliferation was reduced after 24, 48, 72 h to 99, 91 and 72%, respectively, after irradiation with 2 Gy, and it was reduced to 87, 45 and 29%, respectively, after irradiation with 5 Gy, and it was reduced to 55, 27 and 20%, respectively, after irradiation with 10 Gy. On the basis of these results, we determined the 10 Gy dose as the half inhibitory dose at 24 h (ID_{50}), and this dose was selected to study the methylation changes of 22 key cell cycle regulating genes.

3.3. Effect of ionizing radiation on the cell cycle

The effect of ionizing radiation on cell cycle of the 1321NI cells as determined by PI staining and flow cytometry revealed that 70% of cells were clearly arrested in the G2/M phase after 24 h of receiving a 10 Gy dose (Fig. 3).

Table 1
Primer sequences used for quantitative real-time PCR in this study.

Gene symbol	Primers sequence (5'-3')	Reference
CCNE1	F-AAGGTTTCAGGGTATCAGTGGT R-TGGCTTTCTTTGCTCGGGCTT	[81]
CCNF	F-CAGCTGCAGAGGACTCACAA R-CCTCCTCAAACAGGTCATGG	[82]
RAD9A	F-GAGAAGACGGTGGAAAAATG R-GACTCACAACCTGTCCTTCC	[83]
GAPDH	F- ATGACCCCTTCATTGACC R- GAAGATGGTGATGGGATTTC	[84]

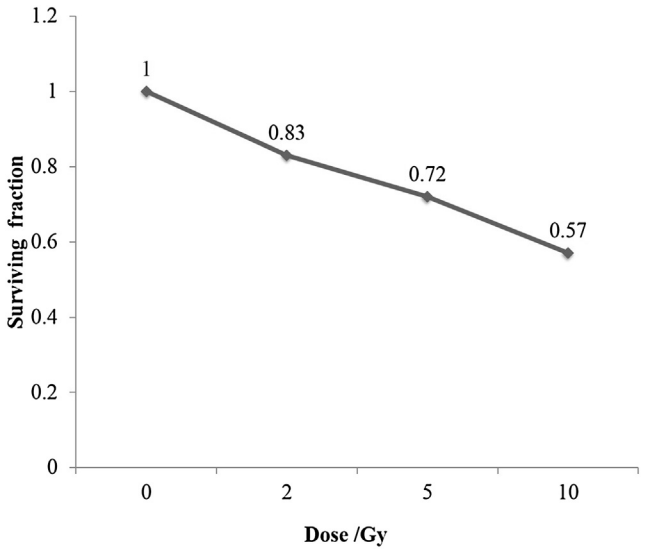

Fig. 1. Effect of ionizing radiation on survival of 1321NI cells. Cells were irradiated by 2, 5 and 10 Gy of gamma radiation and the surviving fraction was measured by MTT assay. Note the dose dependent decrease in cell survival.

3.4. Methylation levels of key cell cycle genes in 1321NI cells

The promoter methylation levels of 22 selected cell cycle regulating genes as determined by Epitect Methyl qPCR Arrays, (Table 2), showed that only 3 out of 22 genes have high methylation levels. The other 19 genes showed methylation levels that was below 0.45%. The methylated genes were *CCNE1*, *CCNF* and *RAD9A*, with 99.69%, 57.06% and 74.03% promoter methylation levels, respectively.

3.5. Azacytidine treatment increases the expression of CCNF, RAD9A and CCNE

To test whether hypermethylation of *CCNF*, *RAD9A* and *CCNE* genes in 1321NI cells affects their expression, we treated 1321NI cells with the demethylating agent azacytidine and measured changes in mRNA levels. As shown in Fig. 4, azacytidine treatment

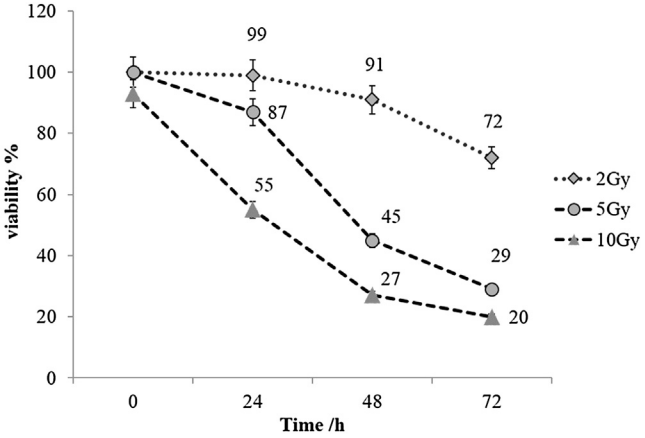

Fig. 2. Effect of ionizing radiation on viability of 1321NI cells. Cells were treated with different doses of ionizing radiation and a MTT assay was carried out after 0, 24, 48 and 72 h. Untreated control samples were assigned a value of 100% and the results of all treatments were compared to untreated cells. Data are expressed as the mean ± SD.

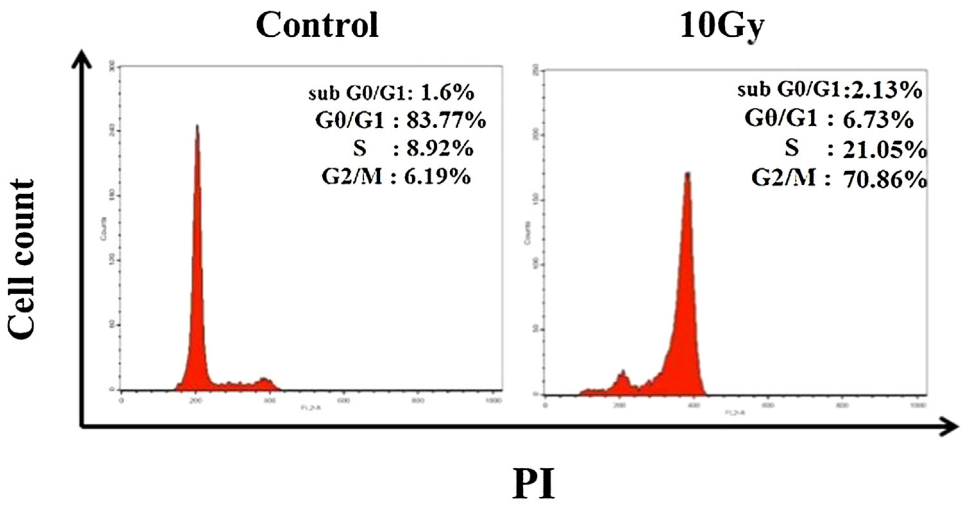

Fig. 3. Effect of ionizing radiation on cell cycle distribution of 1321NI cells. Flow cytometry histograms showed that 70% of irradiated cells were arrested in G2/M phase compared with the non-treated cells.

increased the expression of *CCNE, CCNF* and *RAD9A* by 4.2, 3.5 and 2.5 fold, respectively. This suggests that DNA methylation may be essential in the regulation of the expression of these genes in this cell line.

3.6. Expression of CCNF and RAD9A genes is downregulated by DNA hypermethylation after irradiation

Changes in gene expression of *CCNF*, *RAD9A* and *CCNE1* after 10 Gy irradiation as determined by qPCR showed that *CCNF* and *RAD9A* expression decreased by 4 fold and 2 fold, respectively, while the expression of *CCNE1* did not change compared to the control (Fig. 5).

To test whether irradiation induced changes in the expression of *CCNF* and *RAD9A* genes due to changes in DNA methylation, we compared methylation levels of these genes between 10 Gy irradiated and non-irradiated 1321NI cells. As shown in Table 2, the methylation levels of *CCNF* and *RAD9A* were increased by 3.59% and 3.62% respectively. Interestingly, *CCNE1* methylation level was not affected by irradiation, which is consistent with unchanged expression of this gene after irradiation. Methylation levels of the remaining 19 genes did not change after irradiation.

4. Discussion

Ionizing radiation is used as a treatment option in cancer therapy [29,30]. Glioma patients are routinely treated with gamma radiation, which can extend survival no more than several months. This is due to the radioresistance character of gliomas [3,4]. In this study, we report results that aimed towards a better understanding of the response of 1321NI astrocytoma cells to ionizing radiation and a better characterization of the methylation status of 22 cell cycle regulating gene of 1321NI cells.

Table 2
DNA methylation levels of 22 cell cycle genes in 10 Gy irradiated and non-irradiated 1321NI cells. DNA methylation was assessed using Epitect Methyl qPCR Arrays. Methylation status is represented as percentage unmethylated (UM) and methylated (M) fractions.

Gene name	Gene symbol	0 Gy		10 Gy	
		M%	UM%	M%	UM%
Ataxia telangiectasia mutated	ATM	0.00	99.94	0.06	100.00
Breast cancer 1, early onset	BRCA1	0.12	99.79	0.21	99.88
Breast cancer 2, early onset	BRCA2	0.00	100.00	0.00	100.00
Cyclin B1	CCNB1	0.09	99.88	0.12	99.91
Cyclin D1	CCND1	0.00	100.00	0.00	100.00
Cyclin E1	CCNE1	99.72	0.31	99.69	0.28
Cyclin F	CCNF	60.65	42.94	57.06	39.35
Cyclin-dependent kinase 2	CDK2	0.07	99.85	0.15	99.93
Cyclin-dependent kinase 4	CDK4	0.00	100.00	0.00	100.00
CDK5 regulatory subunit associated protein 1	CDK5RAP1	0.01	100.00	0.00	99.99
Cyclin-dependent kinase inhibitor 1A (p21, Cip1)	CDKN1A	0.45	99.55	0.45	99.55
Cyclin-dependent kinase inhibitor 1B (p27, Kip1)	CDKN1B	0.04	99.86	0.14	99.96
CHK1 checkpoint homolog (S. pombe)	CHEK1	0.00	100.00	0.00	100.00
CDC28 protein kinase regulatory subunit 1B	CKS1B	0.05	99.91	0.09	99.95
Growth arrest and DNA-damage-inducible, alpha	GADD45A	0.19	99.88	0.12	99.81
Minichromosomemaintenance complex component 2	MCM2	0.02	99.95	0.05	99.98
Minichromosome maintenance complex component 4	MCM4	0.01	99.93	0.07	99.99
MRE11 meiotic recombination 11 homolog A (S. cerevisiae)	MRE11A	0.11	99.93	0.07	99.89
RAD51 homolog (S. cerevisiae)	RAD51	0.07	99.93	0.07	99.93
RAD9 homolog A (S. pombe)	RAD9A	77.65	25.97	74.03	22.35
Retinoblastoma-like 1 (p107)	RBL1	0.00	99.94	0.06	100.00
Tumor protein p53	P53	0.09	99.93	0.07	99.91

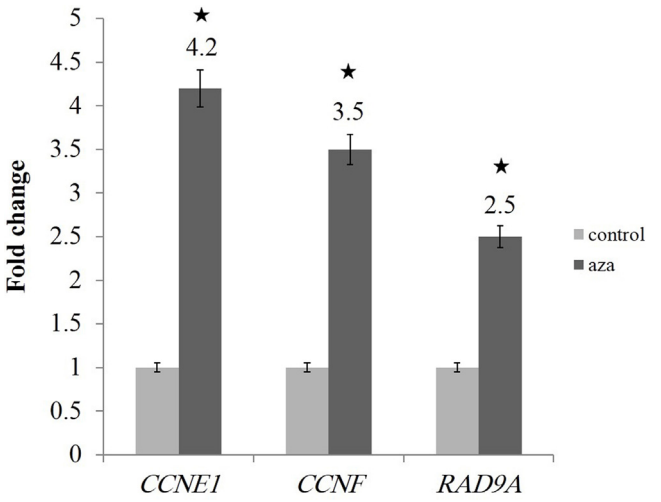

Fig. 4. Gene expression changes of *CCNE1*, *CCNF* and, *RAD9A* after treatment with of azacytidine. qPCR validation of gene expression showed a significant up-regulation of *CCNE1*, *CCNF*, *RAD9A* in 1321NI cells treated with azacytidine compared with control. Data are expressed as the mean ± SD, *P < 0.05.

We used MTT assay to measure cell survival of irradiated cells. MTT assay is a precise method, which had been developed to measure cell survival. Many recent studies used MTT assay because of its high sensitivity, low cost, and the short-term required to conduct cell survival test. Our results showed that the 1321NI cell line is highly radioresistant (SF2 = 0.8 > 0.5). Several studies used SF2 to predict the radiosensitivity of cancer cells [27,31]. Furthermore, we observed a significant dose-dependent decrease in the surviving fraction (SF) with increased doses of radiation, which indicates that the radiation treatment was efficient. Consistent with previous studies on glioma cell lines [32–35]. Our results showed that ionizing radiation inhibits proliferation and decreased the viability of 1321NI cells. Furthermore, we found that 10 Gy dose was the half inhibitory dose (ID_{50}), and we used it further to study promoter methylation changes of 22 key cell cycle regulating genes.

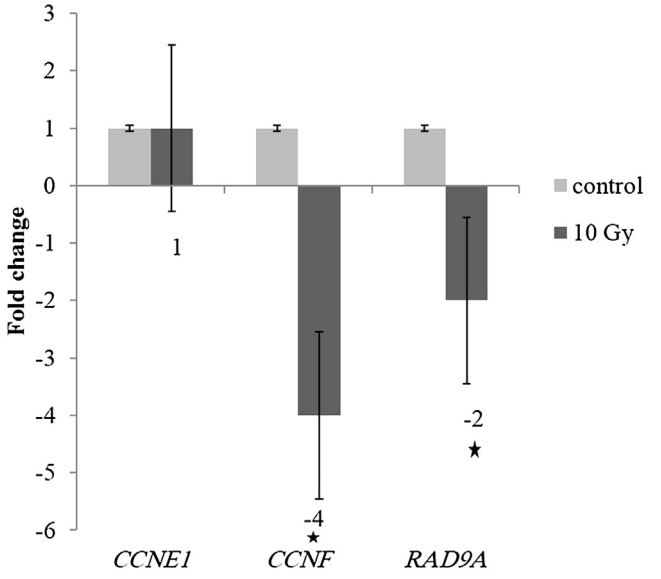

Fig. 5. Gene expression changes of *CCNE1*, *CCNF* and *RAD9A* after irradiation with 10 Gy. qPCR validation of gene expression showed a significant down-regulation of *CCNF* and, *RAD9A* in 1321NI cells treated with 10 Gy, whereas gene expression of *CCNE1* was not changed compared with control. Data are expressed as the mean ± SD.*P < 0.05.

The experiments were designed so the above studies to be performed for the cells before and after irradiation with a single dose of irradiation (10 Gy) which resulted to a G2/M cell cycle arrest. It is known that cell cycle arrest is a consequence of DNA damage induced by irradiation. This arrest gives the cell time to repair the damage. However, when damage is irreparable cells undergo programmed cell death [32–35]. Ionizing radiation leads to several types of DNA damage, including purine lesions, pyrimidine lesions, abasic sites, single-strand breaks (SSB) and double-strand breaks (DSB) [36]. These lesions can be isolated, as well as clustered (formed over a few nanometres) [37] even with low doses like 1 Gy [38]. The yield of DSB (the most lethal damage) increases linearly with radiation dose, starting from a dose of a few mGy [39]. Furthermore, the complexity of clustered DNA damage increases with dose [38]. It has been suggested that clustered DNA damage is involved in the adverse biological effects of ionizing radiation [40]. Clustered damaged DNA sites, which are structurally and chemically complex and include in most cases a DSB, have reduced repairability when compared to that of individual lesions [38,41]. Therefore, clustered damaged sites contribute greatly to effective killing of tumor cells by ionizing radiation. In our study, the viability of 1321NI irradiated cells was decreased as a consequence of the increasing doses (2, 5, and 10 Gy), as shown in Fig. 2. This linear response of the decrease of viability may reflect the accumulation of DNA damage lesions and the complexity of clustered DNA damage, leading to increased cell killing. Deep investigation of the type of clusterd lesions and their biological consequences in 1321NI cells would be an interesting aim of the future study.

Generally, after DNA damage occurs, DNA damage pathways are activated, leading to cell cycle arrest [42]. The apoptotic pathway is initiated if DNA repair is not successful [32–35]. After a 10 Gy irradiation, we found that more than 70% of 1321NI cells were arrested at G2/M phase. This is consistent with previous studies reporting G2/M arrest in glioma cell lines (U251 MG, U343MG-a, and U87MG) after irradiation [35,43,44]. The G2/M check point protects cell viability by allowing time for DNA repair. Mammalian cells repair DSB mainly by two ways: homologous recombination (HR), which is thought to rely on the presence of an intact sister chromatid during S and G2 phase, and the more error-prone non homologous end joining (NHEJ) [45]. Previous studies demonstrated that abrogation of the G2/M checkpoint may cause failure of DNA damage repair, leading to increased radiosensitivity [46]. In addition, epigenetic studies showed that changes in DNA methylation of cell cycle regulating genes affects cell fate after radiation exposure [26,47–49]. Kumar et al. found that DNA methylation status of *ATM* (Ataxia telangiectasia mutated) and *p16* (*CDKN2A*) genes was altered upon radiation exposure, resulting in increased transcriptional activity and possibly influencing radioresistance of cancer cells [24]. It is well established that irradiation induces changes in gene methylation, altering response of cancer cells to radiotherapy [25,50]. Our results showed that the majority (19/22) of the promoters of key cell cycle regulating genes were unmethylated, with the exception of *CCNE1*, *CCNF* and *RAD9A* genes. Previous studies showed that methylation status and protein levels of *CCNF*, *RAD9* and *CCNE1* are prognostic biomarkers in ovarian cancer, hepatocellular carcinoma and breast cancer [51–53].

The CCNE1 protein (Cyclin E) is known to control G1/S transition by forming complexes with Cdk2, leading to inactivation of the latter [54]. *CCNE1* is part of the RB (retinoblastoma tumor suppressor) pathway which directly controls *BRCA* expression involved in DNA repair [55]. Previous studies showed that, upon radiation exposure, *CCNE1*and *CDK2* genes were downregulated due to the p53-dependent induction of *CDKN1A*, enforcing the G1 checkpoint response [56,57]. Therefore, *CCNE1* repression is

considered as an important indicator of G1 arrest. We did not detect any changes in *CCNE1* expression level nor its DNA methylation status after irradiation. This is consistent with our cell cycle results, showing that, after irradiation, 1321NI cells were arrested in G2 and not G1.

On the other hand, we found that the *CCNF* promoter was hypermethylated in the 1321NI cell line, similar to advanced human ovarian cancers [53] and in hepatocellular carcinomas [58]. The *CCNF* gene encodes a member of the cyclin family important to maintain G2 arrest after DNA damage. The CCNF protein is a positive regulator of the nuclear localization of cyclin B1, whose presence in the nucleus is essential for G2/M transition [59]. It is known that ionizing radiation can induces G2/M arrest through a mechanism involving *CCNF* gene repression [60]. Moreover *CCNF* controls DNA repair. After DNA damage, *CCNF* is downregulated in an ATR-dependent manner to allow accumulation of RRM2 which catalyzes the conversion of ribonucleotides to deoxyribonucleotides (dNTPs) necessary for DNA repair and synthesis. Defective elimination of *CCNF* delays DNA repair and sensitizes cells to DNA damage [61]. We observed an increase (about 3.6%) in *CCNF* DNA methylation after irradiation, concomitant with a 3 fold decrease in its transcription. This is consistent with our cell cycle results, showing that, after irradiation, 1321NI cells were arrested in G2/M.

Finally, we found that the *RAD9A* promoter was hypermethylated in the 1321NI cell line similar to what is found in MCF-7 breast cancer cell line and some breast and prostate tumors, although the biological significance of this methylation is unknown [62]. *RAD9* is important for promoting resistance to DNA damage. *RAD9* was implicated in a broad range of processes, including induction of apoptosis, regulation of cell cycle checkpoints, upregulation of *p21*, and binding and stimulating several DNA repair proteins [63–68]. *RAD9* was shown to play a role in several cellular processes, including G2/M checkpoint [69], telomere stability, S- and G2-phase-specific cell survival, and DNA repair, especially in HR (homologous recombination) repair. In fact, *RAD9* influences the activity of proteins that intervene in HR repair occurring predominantly in S and G2 phase cells [70]. It is known that *RAD9* inactivation results in enhanced cell killing after radiation exposure [71].

We observed an increase, (about 3.6%), in *RAD9A* DNA promoter methylation level after irradiation, concomitant with a 2 fold decrease in its transcription, similar to the results of Ma et al. [35], who studied transcriptome changes in the U251 MG glioma cell line irradiated with 7 Gy. They observed that genes involved in DNA repair pathways, including recombinational repair, base excision repair, mismatch repair and nucleotide excision repair, showed systematic repression, indicating the inefficiency of DNA repair after 7 Gy irradiation [35]. It is still unclear, why the expression of *RAD9A* decreases after irradiation. Previous work investigated differential gene methylation after irradiation of cancer cells. Halvorsen et al. used Illumina arrays to investigate DNA methylation changes in breast tumors following 10–24 Gy irradiation. They found 82 differentially methylated genes involved in cellular immune response, proliferation and apoptosis [50]. On the other hand, fractionated irradiation of breast cancer cells (MCF-7) caused differentially methylation of genes involved in apoptotic signaling, and regrowth of cells was also observed [72]. Hypermethylation of *RAD23B* (*P58*), a nucleotide excision repair gene, and *DDIT3*a regulator of cell growth, differentiation and apoptosis, was observed after chronic irradiation of experimental animals, and this hypermethylation was concomitant with transcriptional repression [73]. Similarly, Antwih et al. [25] identified differentially methylated genes in an irradiated breast cancer cell line (MDA-MB-231) after a single dose of 2 and 6 Gy irradiation. They suggested that these genes were involved in cell cycle regulation, DNA repair and apoptosis pathways [25].

Our results showed that irradiation induced an increase in *CCNF* and *RAD9A* DNA methylation. Although these increases were rather small (roughly 4%), they were accompanied by a significant transcriptional repression (roughly 3 fold). Similarly small changes in methylation, accompanied with considerable change in gene expression have been observed in various cancer cell lines after irradiation [25,50,72]. For example, Antwih et al. found that 2 Gy irradiation of MDA-MB-231 cells increased *RB1* methylation by 5%, accompanied with a significant decrease in gene expression (about 3 fold) [25]. Halvorsen et al. used pyrosequencing to study methylation changes in breast cancer cell lines after irradiation with total dose of 6 Gy, and the methylation changes were generally small (for example *H2AFY* gene methylation increased only by 1.8%) [50]. Furthermore, *TRAPPC9* methylation increased only by 6 % after 10 Gy irradiation of MCF-7 cells, accompanied by transcriptional repression [72]. Demethylating agent azacytidine is utilized in numerous studies to demonstrate the relationship between absence of gene expression and gene silencing by DNA hypermethylation [74–78]. We used this agent to validate our results by linking methylation changes to gene expression. We found that the expression of all three methylated genes (*CCNF*, *RAD9*, *CCNE1*) was up-regulated after treatment with azacytidine, indicating that their expression is regulated by DNA methylation. Therefore, it is possible that the decreased expression of *CCNF* and *RAD9* after irradiation was caused by DNA hypermethylation. Similarly, Nandakumar et al. [79] observed that promoter hypermethylation in ultraviolet (UV)-exposed skin and UV-induced skin tumors of mice was associated with decreased mRNA expression of *RASSF1A* (Ras-Association Domain Family 1A) and *p16* (*p16INK4a*/*CDKN2A*) tumor suppressor genes [79]. In another study, it was hypothesized that the *DR4* gene (tumor necrosis factor related apoptosis inducing ligand (TRAIL) receptor) might be silenced by hypermethylation in HN3 laryngeal cells after long-term irradiation, and this epigenetic silencing may be the cause of TRAIL resistance in laryngeal carcinomas treated with irradiation [80].

We studied changes in promoter methylation of 22 cell cycle genes 24 h after one single dose irradiation (10 Gy) of 1321NI cells. More than 70% of irradiated cells were arrested at G2/M cell cycle phase. The majority (19/22) of studied genes were not methylated (0%) and did not show any increase in methylation after irradiation. Therefore, promoter methylation of these 19 genes may not be involved in the regulation of these genes and may not have a role in G2/M cell cycle arrest. Nevertheless, expression of these unmethylated genes could be affected by irradiation through other mechanisms. In fact, we have looked at mRNA expression changes of the studied cell cycle genes, and observed substantial changes in mRNA levels for the majority of the 22 cell cycle genes (data not shown), especially genes implicated in G2/M cell cycle arrest. This indicates no implications for promoter methylation of these genes. In contrast, promoter methylation seems to play an important role in regulation of *CCNF* and *RAD9*, the repression of which favors the cell cycle arrest at G2/M phase.

5. Conclusions

We studied the 1321NI human astrocytoma cells response to different doses of ionizing radiation. We found that 1321NI cells is highly radioresistant. Ionizing radiation inhibited proliferation and caused G2/M cell cycle arrest. Irradiation of 132NI cells with a 10 Gy dose increased the promoter methylation of *CCNF*, *RAD9* and *CCNE1* genes out of selected 22 key cell cycle regulating genes. Ionizing radiation increased DNA methylation in *CCNF* and *RAD9* genes, involved in G2/M cell cycle arrest and check point regulation. Azacytidine treatment confirmed that expression of *CCNF* and *RAD9* is regulated by methylation. Hypermethylation of *CCNF* and *RAD9* observed in 132NI irradiated cells was associated

with decreased mRNA expression. Further studies combining methylation profiling and gene expression of DNA damage (especially DNA repair) pathways, are needed to elucidate the impact of radiation treatment on cancer cells. These studies must take into account time dependence and the effect of total dose fractionation in order to simulate irradiation of real tumors. A better understanding of radioresistance at the molecular level could help in identifying targets for radiosensitizing tumors, and in improving radiotherapy in the future.

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Conflict of interests

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