Evaluation of Capability and Relationship of Different Radiobiological Endpoints for Radiosensitivity Prediction in Human Tumor Cell Lines Compared with Clonogenic Survival

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ABSTRACT

Background: Establishing a predictive assay of radiosensitivity (as an appropriate, practical and cost-effective method) has been challenging.

Objective: The purpose of this study is to evaluate the capability and relationship of various endpoints, including GammaH2AX, micronuclei; and apoptosis in determining the human tumor cell lines radiosensitivities compared with clonogenic survival.

Material and Methods: In an experimental in-vitro study, the response of carcinoma cell lines of HN5 and HeLa to 2 Gy of 6 MV photon beam was investigated via various assays.

Results: Survival fraction at 2 Gy (SF2) of HeLa and HN5 was indicated as 0.42 ± 0.06 and 0.5 ± 0.03 respectively, proposing more radioresistance of HN5. This finding was confirmed with "2 Gy apoptosis enhancement ratio" which was 1.77 and 1.42 in HeLa and HN5. The increased levels of DNA DSBs were observed after irradiation; significant in HeLa with enhancement rate of 19.24. The micronuclei formation followed an ascending trend post irradiation; but with the least difference between two cells. Although the relationship between micronuclei and clonogenic survival was moderate ($R^2 = 0.35$), a good correlation was observed between apoptosis and clonogenic survival ($R^2 = 0.71$).

Conclusion: The results of studied endpoints agreed with the SF2, highlighting their capabilities in radiosensitivity prediction. In terms of the enhancement ratio, gammaH2AX foci scoring could be a valid indicator of radiosensitivity but not the exact surrogate marker of survival because no correlation was observed. Moreover, considering the chief determents comprising lack of time and money, the apoptotic induction might be an appropriate indicator with the best correlation coefficient.

Introduction

Recent advances in treatment modalities including surgery, chemotherapy, and radiotherapy have been a huge help for patients undoubtedly. More than 50% of patients experienced radiotherapy during their treatment. Irrespective of its remarkable merits includ-

ing tumor control and survival increase, the development of a second malignant neoplasm could be a main concern [1]. Therefore, due to the radiotherapy-induced adverse effects, the delivery of radiation total dose to the majority of patients could be restricted [2].

In another aspect, since tumor heterogeneity was considered as a chief cause of variation in response to radiotherapy [3], achieving an accurate biomarker to predict the treatment outcome could be of paramount importance. However, finding the relationship between pre-treatment evaluations and total tumor response to radiotherapy has been remained debatable [3]. Consequently, establishing a predictive assay has been an important issue in radiobiology; and radiosensitivity determination could be regarded as an integral part of cancer management [4]. Researchers have made a sustained effort to achieve an appropriate molecular biomarker to increase tumor control probabilities and normal tissue complications decrease; and several endpoints have been introduced to predict radiosensitivity [5]. From the viewpoint of cellular response, the clonogenic survival has been a valid predictive assay and survival fraction at 2 Gy (SF2) has ascertained as a standard method to compare radiosensitivity. However, the use of clonogenic assay in a clinical setting might be restricted due to low plating efficiency of some cell types and the time needed for colony formation [6]. Since the radiation-induced chromosomal damage might result in cell death, accordingly, chromosomal aberration could be considered as a reliable radiosensitivity predictor, and G2 assay for peripheral blood lymphocyte was outlined as an appropriate approach [7, 8]. The contribution of cytogenetic methods for the radiosensitivity manifestations has been stated in this regard [9, 10] and the Cytochalasin-B blocked micronucleus assay was reported as a prevalent technique scoring micronucleus in bi-nucleate cells, indicating chromosomal breakage or loss [11, 12]. Several lines of studies have focused on

the cell survival prediction through the detection of double strand breaks (DSBs); rapid phosphorylation of Ser139 on the specialized histone H2AX (gH2AX) has been indicated as an early and sensitive molecular marker of DNA double strand breaks (DSBs) induction [6, 7]. To find better indicators, some investigations have been conducted to assess the combination of different assays including simultaneous use of gH2AX plus apoptosis [6] or micronuclei accompanied with apoptotic induction [13] and consequently, distinct reports were expressed.

To date, the issue of radiosensitivity and its mechanisms remained controversial and clarifying this ambiguity needed more experimental confirmations. Although number of studies developed to find the predictive way to radiotherapy response, the issue of an appropriate, practical, and cost-effective method has been still remained allusive. Since DNA damages as well as chromosomal aberrations might be distinct causes of cell death, the aim of current study was to evaluate the capability of various endpoints including GammaH2AX, micronuclei, along with apoptosis compared with the clonogenic survival for radiosensitivity prediction in human tumor cell lines. Two different tumor cell lines with distinct radiosensitivities were selected to assess their 2 Gy enhancement ratios quantitatively. This in-vitro study could also make us capable of clarifying the correlation of studied endpoints compared with the standard clonogenic survival assay.

Material and Methods

Cell Culture

In this experimental in-vitro study, two human carcinoma cell lines of head and neck squamous cell carcinoma (HN5) compared with the human cervix carcinoma (HeLa) cell line were selected that had distinct radiosensitivity according to the previous reports. Since head and neck squamous cell carcinoma has

been considered as a radioresistant tumor cell based on various radiobiological parameters [14], comparing its radiation response with less resistant cells could be a sensible analysis. HeLa and HN5 were obtained from the National Cell Bank of the Pasteur Institute (Tehran, Iran) and grown in monolayers in RPMI1640 and Dulbecco's Modified Eagle's (DMEM) (Gibco, Invitrogen, UK) mediums respectively, supplemented with 10% fetal bovine serum (FBS, Gibco, Invitrogen, UK) and 1% penicillin-streptomycin (Gibco, Invitrogen, UK). Cultures were maintained in humidified atmosphere of 95% air/ 5% CO_2 at 37 °C.

Irradiation Setup

Exponentially growing cells were seeded in T-25 culture flasks containing 5 ml culture medium 48h prior to irradiation treatment in triplicate manner. Irradiation was performed at room temperature with a dose of 2 Gy by a 6 MV photon beam using Varian 2100C linear accelerator (Linac) with field size of 20 × 20 cm² at the isocenter. PTW water equivalent slab phantoms with total thickness of 2 cm at the top and 6 cm under the cell dishes were placed to obtain SSD (Source to Skin Distance) of 100 cm. The Linac has been calibrated in terms of the IAEA TRS 398 dosimetry protocol.

Cellular Evaluation by Clonogenic Assay

The clonogenic survival assay as a standard method has been used prevalently [15]. Briefly, exponentially growing cells were irradiated with dose of 2 Gy at room temperature using a megavoltage (6 MV) X-ray radiation unit according to the setup described in the irradiation section. Cells were washed with phosphate-buffered saline (PBS), trypsinized, counted using "Trypan Blue" dye (Sigma- Aldrich, USA), and then the sparsely definite numbers of appropriate densities, which were proportional to the radiation doses, were seed-

ed in six-well plates and incubated 10-14 days seeded. Following plating and 2-week growth in the humidified 37 °C incubator, the colonies (>50 cells) formed and were stained with 0.5% crystal violet (Sigma- Aldrich, USA) and counted using light microscope (CETI, Belgium). Each experiment usually was repeated two to three times. Plating efficiency percentage (%PE) explained as the ratio of the number of counted colonies to the seeded cells multiplying by 100. Survival Fraction (SF) also calculated by normalizing efficiencies of the irradiated groups to the unirradiated controls and SF2 defined as the survival fraction at 2 Gy. The "2 Gy clonogenic cell death enhancement ratio" of HeLa and HN5 was defined as the PE of the untreated control cells to the value obtained from 2 Gy-irradiated ones.

Apoptosis Evaluation by Flowcytometry

The HeLa and HN5 cells were seeded into six-well plates at the density of 2×10^4 cells/ well and incubated for 24 h. Afterwards, they were exposed by dose of 2 Gy of the 6-MV X-ray beam and were preserved in the 37 °C incubator. Annexin V-FITC Staining Assay was performed to assess apoptosis at the specific time of 24 h after irradiation as an apoptosis induction appropriate interval [16]. The apoptotic assay was initiated using FITC Annexin V Staining Kit (BioLegend), in accordance with the manufacturer's instruction. Briefly, after incubation, the supernatant medium comprising floating cells was transferred to the falcon tube. Adherent cells were trypsinized and added to the preserved medium. The cells were then centrifuged, counted, and washed twice with PBS. 5×10^5 cells were resuspended in 200 microliters of binding buffer (1×), and five microliters of Annexin V- FITC was added to each sample and then incubated for 15 min at room temperature in the dark; which was followed by 10 microliters of PI (20 μg/ml) addition. Samples were analyzed for the apoptotic and necrotic cells presence

by the use of BD FACS Calibur flow cytometer (BD Bioscience). 10,000 calls per each sample were evaluated and the obtained data were analyzed using the BD Cell Quest Pro software. For better analysis, the "2 Gy apoptosis enhancement ratio" was calculated to compare the results of both cell lines quantitatively defined as the apoptotic percentage of 2 Gy-irradiated cells to the value obtained from the untreated control ones.

DNA Damage Evaluation by GammaH2AX Foci Assay

Immunofluorescence technique of Gamma-H2AX Foci assay has been well documented as an early sensitive indicator of DSBs [17]. Experimental procedures of the assay were based on the described protocol of Rothkamm and Lobrich (2003) with some modifications regarding time and concentrations [18]. Considering the optimum time point for gH2AX foci scoring when the size and intensity of the majority of induced foci were appropriate for valid counting [19], cell harvesting was performed 1h after the irradiation. Briefly, 2×10^5 cells were seeded into a slide and fixed with cold 4% formaldehyde for 20 min which followed by PBS washing three times. Premeabilization was performed with 0.25% Triton-X-100 and then blocked with 1% bovine serum albumin (BSA) accompanying with 0.05% Tween20. At the next stage, cells were incubated with an anti-phospho-histone H2AX antibody (Millipore) at 1:500 dilutions under wet chamber circumstances for 2h, and then washed three times with BSA. Afterwards, cells were incubated with Anti-Mouse IgG FITC antibody (Sigma- Aldrich, USA) at 1:500 dilutions in a dim lighted wet chamber for 45 min which was followed by three-time PBS washing. The cells were then stained with Dapi (Abnova, Taiwan), and the foci scorings were implemented by eye under Olympus Fluorescence microscope with U/B/G, FITC, TXRED, DAPI filters in a meticulous care. Each experiment involved at least three inde-

pendent slides and usually experiments were repeated at least two times. The "2 Gy GammaH2AX enhancement ratio" of HeLa and HN5 was defined as the foci per cell (FPC) of 2 Gy-irradiated cells to the value obtained from the untreated control ones.

Cytogenetic Evaluation by Micronucleus Assay

The HeLa and HN5 cells were seeded into culture flasks at the density of 1×10^6 cells and exposed by the dose of 2 Gy of the 6-MV X-ray. The Cytokinesis-Blocked Micronucleus assay (CBMN) is the standard cytogenetic method scoring micronucleus in bi-nucleate cells which the cytochalasin-B (Sigma-Aldrich, USA, 6 mg/ml final concentration) treatment as an inhibition of the cytokines fulfillment was performed 1h post irradiation. By passing 24 h from treatment with cytochalasin-B, cell harvesting was performed by trypsinization (0.25% trypsin-EDTA, Gibco-BRL) for 5 min at 37 °C. The samples were centrifuged 1200 rpm for 7 min, the supernatant was discarded and cells were then subjected with prefix solution comprising fixative (Methanol: Acetic Acid, 6:1 v/v) plus hypotonic solution (KCl, 0.075M). Following the secondary centrifuge, washing with the cold fixation solution was implemented; and the resuspended fixed cells were dropped on the precooled glass slides to achieve appropriate cells spreading. After drying at room temperature, the slides were then stained in Giemsa 10% (Sigma-Aldrich, USA) and the rate of micronucleus (MN) was counted under light microscopy (Nikon, YS100, Japan) in a way that 500 bi-nucleate cells were scored for each sample according to the Fenech's criteria [20] at ×40 magnification. The "2 Gy micronuclei enhancement ratio" of HeLa and HN5 was defined as the micronuclei frequency of 2 Gy-irradiated cells to the value obtained from the untreated control ones.

Statistical Analysis

All data were expressed in terms of mean

values ± SEM (standard error of the mean). The correlation between each pair of factors was evaluated using Spearman correlation (Since they did not follow normal distribution) and Spearman correlation coefficient was determined. Statistical analysis was performed using SPSS software (version 24). The P-values of less than 0.001 (***), 0.01 (**), and 0.05 (*) were considered as a significant level.

Results

Cellular Evaluation by Clonogenic Assay

Survival fraction at 2 Gy (SF2) of HeLa and HN5 was indicated as 0.42 ± 0.06 and 0.5 ± 0.03 respectively, proposing more radioresistance of HN5. The acquired results indicated the descending rate of PE in 2 Gy-irradiated cells compared with the untreated controls which was statistically significant difference (P<0.05) (Figure 1). The "2 Gy clonogenic cell death enhancement ratio" of HeLa and HN5 was 2.36 and 1.98, respectively.

Apoptosis Evaluation by Flowcytometry

The obtained results demonstrated an increase in apoptotic rate of irradiated cells compared with the untreated control group which was statistically significant (P<0.05) (Figure 2). The findings of HeLa and HN5 revealed the "2 Gy apoptosis enhancement ratios" of 1.77 and 1.42, respectively, which were an indicator of more apoptotic cell death in radiosensitive cell line. Noteworthy, all compared values of necrosis were almost less than 3%; and consequently, considered as negligible.

DNA Damage Evaluation by GammaH2AX Foci Assay

GammaH2AX foci per cell (FPC) scored qualitatively, and were also processed by Image J software1.50i (Figure 3). The FPC ascending trend was obvious from the control cells to the 2 Gy-irradiated ones in both cell
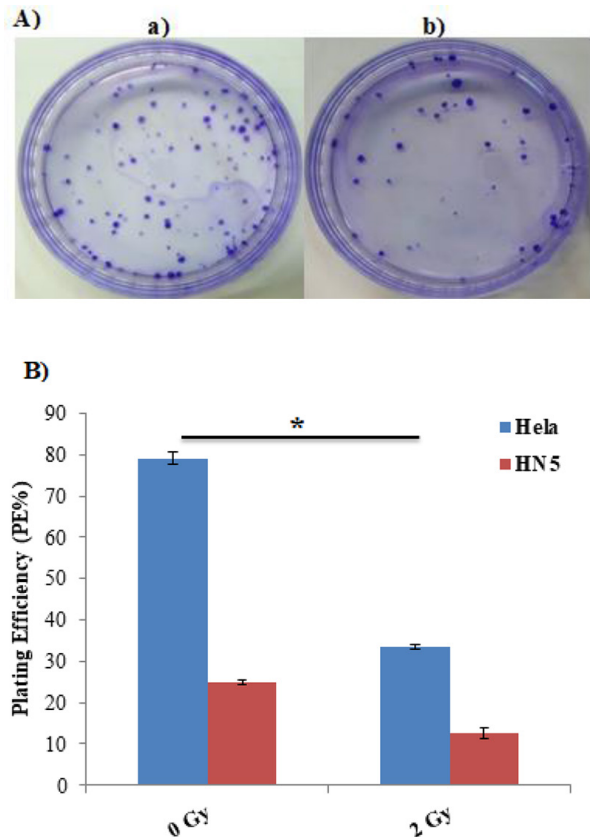

Figure 1: A) Samples of clonogenic formation stained with crystal violet; a) Control of HeLa; b) Control of HN5; B) Plating efficiencies Plating efficiency percentage (%PE) in HeLa and HN5 after 2 Gy X irradiation, * P < 0.05.

lines, which indicated the increased number of DNA DSBs after irradiation; and this observed difference was also statistically significant. Moreover, the HeLa and HN5 "GammaH2AX enhancement ratios" indicated the value of 19.24 and 13.38, respectively.

Cytogenetic Evaluation by Micronucleus Assay

The number of micronuclei (MN) as a cytogenetic endpoint in the studied cells followed an upward slope after receiving 2 Gy X-irradiation (Figure 4); and the observed difference was also statistically significant (P< 0.001). The "2 Gy micronuclei enhancement ratios" of
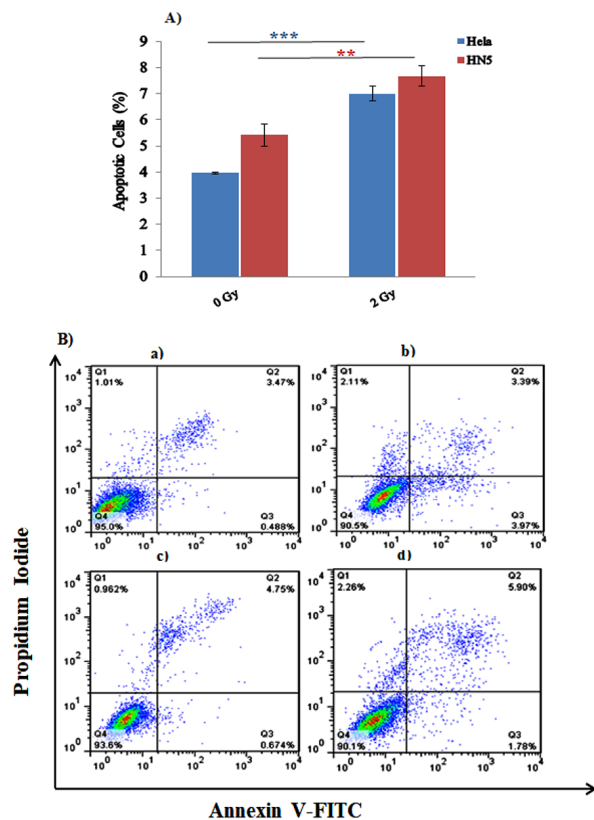
Figure 2: A) Flowcytometry of apoptosis induction in cell lines of HeLa and HN5 based on Annexin V-fluorescein isothiocyante (FITC) and Propidium Iodide (PI) double staining (Data expressed as mean ± SEM (Standard Error of the Mean) of three independent experiments), **P < 0.01 and ***P < 0.001; and B) Scatter plots of apoptosis in a) HeLa 0 Gy, b) HeLa 2 Gy, c) HN5 0 Gy, d) HN5 2 Gy.
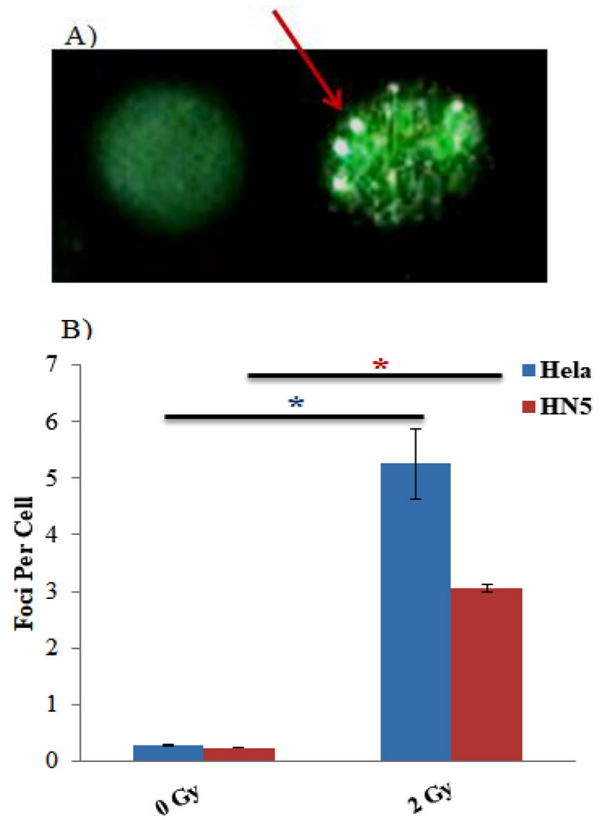
Figure 3: A) GammaH2AX foci scored using fluorescence microscope under fluorescein isothiocyante (FITC) filter in HN5 after 2 Gy irradiation; examples of intact cell without foci and single cell (red arrow) with different foci; B) Foci Per Cell (FPC) scored in HeLa and HN5 cell line after 2 Gy X irradiation (Data expressed as mean ± SEM (Standard Error of the Mean) of three independent experiments), *P< 0.05.

HeLa and HN5 were 3.078 and 2.95, respectively. All obtained "2 Gy enhancement ratios" of different endpoints are presented in Table 1.

Relationship between different endpoints and Clonogenic Survival

The correlation of the histone gH2AX foci and the clonogenic survival for the studied cell lines were assessed which was not statistically significant. The relationship between the number of micronuclei and the clonogenic survival was moderate ($R^2 = 0.35$); but a good correlation was observed between the apoptosis and the clonogenic survival for the studied cell lines ($R^2 = 0.71$).

Discussion

In the current work, different endpoints of gH2AX foci, micronuclei, apoptosis and clonogenic survival were studied to compare their capabilities in demonstrating the difference of radiosensitivity in two different human tumor cell lines. Since head and neck squamous cell carcinomas have been considered as the
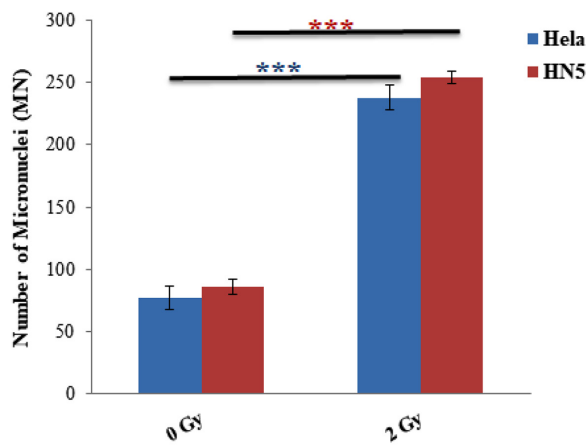
Figure 4: Micronuclei frequency scored in different HeLa and HN5 after 2 Gy X-irradiation (Data expressed as mean ± SEM (Standard Error of the Mean) of three independent experiments), ***P < 0.001.

Table 1: "2 Gy Enhancement Ratios" of different studied biomarkers in two tumor cell lines.

HN5	HeLa	
1.98	2.36	2 Gy Clonogenic Cell Death ER
1.42	1.77	2 Gy Apoptosis ER
13.38	19.24	2 Gy GammaH2AX ER
2.95	3.078	2 Gy Micronuclei ER

ER describes enhancement ratio of biomarkers after 2 Gy irrdiation compared to untreated control cells

aggressive, genetically complex and radioresistant cancers [14, 21], consequently we selected head and neck squamous cell carcinoma (HN5) cell line to compare with less resistant cells i.e. human cervix carcinoma (HeLa). Because SF2 has been determined as the standard method for radiosensitivity prediction, it seems crucial to assess different endpoints after the dose of 2 Gy X-irradiation. To obtain quantitative analysis, the "2 Gy enhancement ratios" of different studied biomarkers were calculated by normalizing the values of the

molecular, cytogenetic and cellular endpoints after 2 Gy irradiation compared with the value obtained from untreated control cells. Parallel with previous confirmatory investigations, the resultant SF2 of the current work indicated that HN5 could be considered as the radioresistant cell line. Moreover, the attained data of other studied endpoints of this investigation were in line with this observation.

Given the most reliable assay for monitoring the radiation survival, clonogenic assay could be nominated. However, it has been indicated as a time consuming method which could not be beneficial for the cell types with low cloning efficiencies [10]. Therefore, finding another appropriate clinical endpoint with the least pitfalls could be of paramount importance. Rapid phosphorylation of Ser139 on the specialized histone H2AX (gH2AX) has been considered as an early and sensitive molecular marker of DSBs induction [7]. Since unrepaired DNA DSBs as persistent damages could lead to cell death particularly mitotic ones, consequently gH2AX foci assay could be regarded as a promising approach [22]. It was elicited that gH2AX foci scoring even 24 h post irradiation i.e. "residual damage" could also be considered as a predictive biomarker of tumor response [3]. Several lines of studies have proved that the clonogenic survival results were in accordance with the gH2AX foci scoring [23, 24]. Among different biomarkers of the current work, gH2AX foci exhibited the peak of difference in displaying the radiosensitivity of studied cell lines and had the most enhancement ratio; whereas, the micronuclei exhibited the trough. Based on the correlation study, the relationship between the frequency of gH2AX foci and the clonogenic survival for the studied cell lines was not statistically significant. However, different statistical parameters played the crucial role in this discrepancy that might related to the nature of gamma-H2AX foci. Remarkable variation in gH2AX formation has been reported particularly in the first hour after irradiation [19]. Kunogi et

al. investigated the correlation of frequency of gH2AX foci and the clonogenic survival; and consequently, they had not reported good relationship [6]. But they attributed this phenomenon to not normalizing the gH2AX foci frequency because after normalization using the DNA content in each cell line, better relationship was observed.

Given another alternative for radiosensitivity predictions, the chromosomal aberration could be outlined. Because chromosomal aberrations were usually tied to misrepair or altered repair function of DNA damages; consequently, they could be also related to the cellular radiosensitivity [5]. Finding correlation between the micronucleus (MN) formation and the clonogenic survival raised a question which was investigated by previous studies, revealing the linear correlation [11, 12]. However, the association between G2 chromosomal radiosensitivity and the genetic predisposition particularly in case of a head and neck cancer was assessed and reported that head and neck cancer patients have a significantly higher mean frequency of chromatid breaks per cell than healthy individuals [8]. Although our results confirmed more "micronuclei enhancement ratio" of HN5, the observed difference between two studied tumor cells was slight. The relationship between the number of the micronuclei and the clonogenic survival was also moderate ($R^2 = 0.35$). Several investigations have attempted to demonstrate that the combination of distinct assays to predict cell survival after irradiation was more successful, but establishing these procedures as too complicated methods for use in a clinical setting made this issue controversial. It has been previously revealed that the combination of the micronuclei (MN) and apoptosis could be the sufficient predictive assays. Moreover, several studies have also reported the paired utility of DNA damages and chromosomal aberrations [12, 25-27]. Additionally, the simultaneous use of the gH2AX foci assay and apoptosis induction have been demonstrated as an appropriate indicator [6].

Although gH2AX foci exhibited the peak of difference in displaying the radiosensitivity of studied cell lines and also the most enhancement ratio in our investigation, this assay involved some problematic issues in clinical setting. The noticeable variation in gH2AX formation might be observed which was due to different factors including cell line, microscope and camera optical characteristics, image analysis, and foci scoring strategies [19]. Additionally, the gH2AX foci assay was considered as an expensive method. Apoptosis might be our second predictive assay due to its appropriate enhancement ratio, particularly considering the time and money allocated to this method. Parallel with the findings of other endpoints, apoptotic cells were enhanced after 2 Gy, which was significant in the radiosensitive cell line. Moreover, good correlation was revealed between the apoptosis induction and the clonogenic survival ($R^2 = 0.71$). However, elucidation of the relationship between apoptosis and the clonogenic survival has been complex because apoptosis could be considered as a mitotic death or/and non-mitotic death consequence [28]; and there is still a big controversy surrounding the apoptosis involvement in the radiation-triggered cell death. Since micronuclei illustrated subtler degree of the radiosensitivity difference between two cell lines compared with SF2, therefore micronuclei might be as the last predictive indicator in our study.

Conclusion

The evaluation of different endpoints indicated their parallel results with the resultant SF2, which highlighted their capabilities in radiosensitivity prediction. However, the utility of DNA damage foci scoring could be intended as a valid indicator of radiosensitivity but not the exact surrogate marker of cell survival because the correlation was not observed. Moreover, considering the chief determents comprising lack of time and money, the apoptotic induction might be an appropriate

indicator with the best correlation reported. For better understanding, this in vitro study should be accomplished with in-vivo tumor models along with human tumor samples of patients to determine their correlation, precisely. Therefore, the clinical useful predictive method of radiosensitivity has still waited for more research in the future.

Acknowledgment

Authors' Contribution

F. Pakniyat conceived the idea. H. Mozdarani made substantial contributions to the design of the study. A. Mahmoudzadeh and S. Gholami helped F. Pakniyat in method implementation. F. Pakniyat drafted the manuscript and HA. Nedaie was responsible for the overall supervision of the work, as a corresponding author. All the authors read, modified, and approved the final version of the manuscript.

Ethical Approval

The Ethics Committee of Tehran University of Medical Sciences approved the protocol of the study (IR.TUMS.REC.1395.2464).

Informed consent

The work was carried out on cell lines and therefore, no participation consent was obtained.

Funding

Conflict of Interest

None

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