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Research Article
Effect of Temporal Pattern of Radiation in Intensity Modulated Radiotherapy on Cell Cycle Progression and Apoptosis of ACHN Renal Cell Carcinoma Cell Line

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Abstract

Background and Objective: The existence of a hypersensitive radiation response to doses below 1 Gy is well established for many normal and tumor cell lines. The aim of this study was to ascertain the impact of temporal pattern modeling IMRT on survival, cell cycle and apoptosis of human RCC cell line ACHN, so as to provide radiobiological basis for optimizing IMRT plans for this disease. **Materials and Methods:** The ACHN renal cell carcinoma cell line was used in this study. Impact of the triangle, V, small-large or large-small temporal patterns in the presence and absence of threshold dose of hyper-radiosensitivity at the beginning of patterns were studied using soft agarclonogenic assays. Cell cycle and apoptosis analysis were performed after irradiation with the temporal patterns. **Results:** For triangle and small-large dose sequences, survival fraction was significantly reduced after irradiation with or without threshold dose of hyper-radiosensitivity at the beginning of the patterns. In all of the dose patterns, cell cycle distributions and the percentage of apoptotic cells at 24 h after irradiation with or without priming dose of hyper-radiosensitivity showed no significant difference. However, apoptotic cells were increased when beams with the smallest dose applied at the beginning of dose pattern like triangle and small-large dose sequence. **Conclusion:** These data show that the biologic effects of single fraction may differ in clinical settings depending on the size and sequence of the partial fractions. Doses at the beginning but not at the end of sequences may change cytotoxicity effects of radiation.

Key words: Temporal pattern, dose sequence, renal cell carcinoma, IMRT, ACHN cell line

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Intensity-modulated radiotherapy is a technique of radiation dose delivery developed to generate dose distribution that can be fit with tumors of a complex geometrical shape while sparing surrounded radiosensitive normal tissues[1-3]. Although, IMRT has proven to be beneficial clinically for numerous tumor sites, there are some radiobiological issues that are not fully understood. The IMRT has been associated with prolonged fraction times (from 5-30 min) depending on the site and complexity of treatment[4]. A number of studies have focused on the effect of prolonged fraction delivery time in IMRT on tumor cell killing[5-7]. Furthermore, the radiobiological outcome of radiotherapy treatment is influenced not only by prolonged fraction times but also by the pattern of dose deposition[8]. In IMRT technique, a tumor can receive multiple Partial Fractions (PFs) within a given fraction[9] and it is possible that some of these PFs will be less than 1 Gy. Altman *et al*.[8] were the first to observe the effect of dose temporal pattern on tumor cell lines. In addition, several recent studies have demonstrated that a relative cell killing at doses below 1 Gy is more than at doses above 1 Gy. This phenomenon has been termed low-dose hyper-radiosensitivity (HRS). Strikingly, this radiosensitivity is more apparent in radioresistant cell lines[10]. Absence of cell cycle arrests in the G1 and G2 phases has been shown to be associated with hypersensitivity of eukaryotic cells to ionizing radiation[11]. In this case, the growth arrest caused by checkpoints allows the cell to repair the damage. Unrepaired or misrepaired DNA damages can lead to cell death[10-12].

Some studies using IMRT have investigated low-dose hypersensitivity with a series of pulses of equal doses[13,14]. However, this is not accurate at a single point within the patient. It means that with different modulation and beam attenuation to that point of each applied field delivering pulses of equal dose is highly unlikely.

Lin and Wu[15] reported a reduced survival fraction if a smaller "Priming" dose precedes a larger dose fraction, compared to the reverse sequence of dose delivery. They attributed the observed effects to a combination of low-dose hypersensitivity and high-dose increased radioresistance but without conclusive proof, the overall treatment time was not controlled and the intervals between partial fractions were not constant.

In this study, it was attempted to ascertain the impact of temporal pattern modeling IMRT on survival, cell cycle and apoptosis of human RCC cell lines ACHN, so as to provide radiobiological basis for optimizing IMRT plans for this disease.

MATERIALS AND METHODS

Cell culture: The ACHN renal cell carcinoma cell line purchased from the Iranian Biological Resource Center was maintained in minimum essential medium in Earl's BSS supplemented with 10% heat inactived FBS, 1% non-essential amino acids, 1 mM sodium pyruvate, 2 mM L-glutamine, 100 μg mL^{-1} streptomycin and 100 U mL^{-1} penicillin. The cell line was incubated at 37°C in 5% CO_2 in air, when the cells reached near 80% confluency they were trypsinized with 0.25% trypsin-EDTA and then subcultured.

Irradiation conditions: All irradiations were carried out using a 6 MV photon beam produced by a clinical linear accelerator (ONCOR, Siemens Company, Germany). A six-well plate containing the ACHN cells was placed in a phantom composed of a rectangular block of solid water with a plate-sized cavity at the center. Five centimeters thick solid water slab was placed at the bottom of the phantom to ensure the full backscatter condition. About 1.5 cm thick solid water slab was placed on the top of the plate to serve as a build-up material for the 6 MV beam. The plates were irradiated using 20×20 cm^2 field size and a dose rate of 3 Gy min^{-1}. *In vivo* diode (QED, Sun Nuclear Company, United State America) dose radiation measurement was performed to ensure the accuracy of delivered dose within ±2%.

Evaluation of the threshold dose of HRS: The cells were harvested from a stock culture and the equal number of cells per well (2000) was plated with soft agar for doses ranging from 0-2 Gy. Then, the colony formation assay was used to determine the threshold dose of HRS.

Effect of temporal pattern on the cell killing: To compare effectiveness of temporal pattern on the survival of ACHN cell line, each plate was irradiated with the triangle (Δ), V, small-large (S-L) or large-small (L-S) dose sequences with or without priming dose of HRS threshold (Fig. 1). For each pattern, a total dose of 10 Gy was divided into six fields with 3 min interval between each partial fraction so fraction duration time was 15 min.

Soft agar colony formation assay: The soft agar colony formation assay was used to acquire the survival fraction of ACHN and to determine the effect of temporal pattern on the cell killing. Cells were plated on top of 1% bottom agar in growth medium and overlaid with 0.3% top agar in growth medium. Cells were fed 2 mL of growth medium every 3-4 days for 4 weeks. Colonies (containing) were stained with a 0.05% aqueous solution of crystal violet and counted.

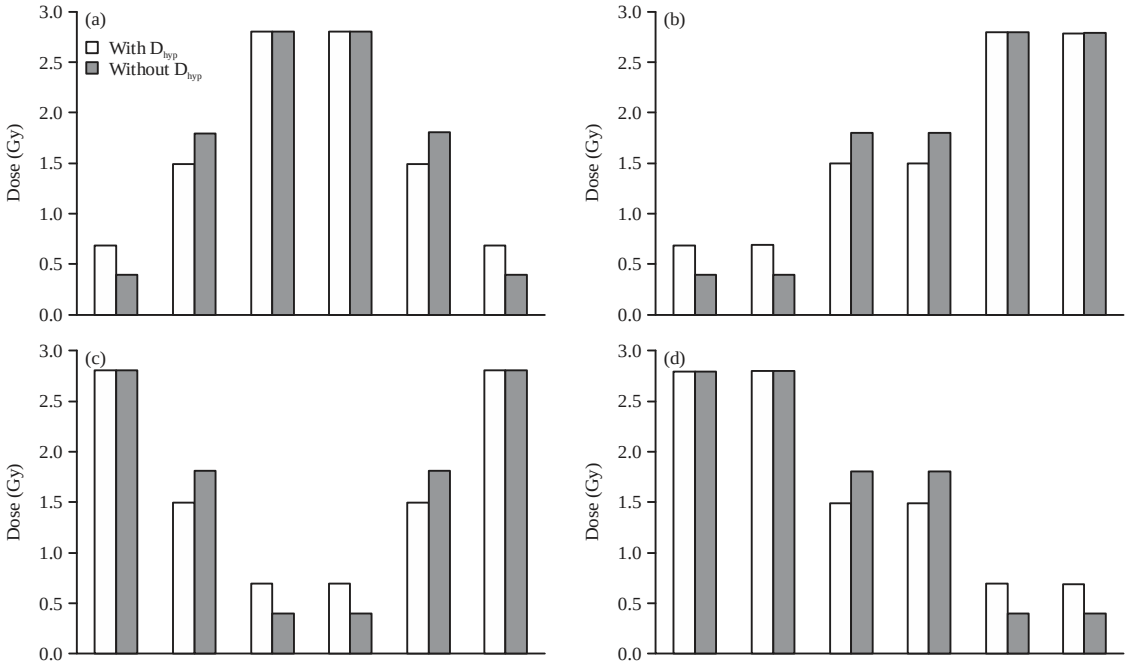

Fig. 1(a-d): Schematic diagram of four dose delivery patterns, (a) Δ pettern, (b) S-L pattern, (c) V pattern and (d) L-S pattern. Each schedule was composed of six-field beam-on components, separated by a beam-off period of 3 min duration

For the unirradiated control, 200 cells were inoculated into a 6-well plate and allowed to grow. The Plating Efficiency (PE) was determined as the percentage of the number of colonies observed to the number of cells seeded. Surviving Fraction (SF) is the ratio of the numbers of colonies produced to the number of cells plated with a correction necessary for PE:

$$\text{Surviving fraction} = \frac{\text{Colonies counted}}{\text{Cells seeded} \times \text{PE}/100}$$

Measurement of the cell survival fraction was repeated three times and the survival data were fitted to the LQ model.

Cell cycle analysis: Twenty-four hours after irradiation the cells with the Δ, V, S-L or L-S dose sequences, they were collected and washed with cold PBS, then, they were kept in precooled 70% ethanol at -20°C for fixation overnight. The cells were stained with 0.1% (v/v) triton X-100 in PBS, 0.2 mg mL^{-1} RNase-free DNase A and 20 μL of 1 mg mL^{-1} PI. After an incubation time of 30 min, the cell-cycle distribution was measured with a FACS can flow cytometer (Becton-Dickinson).

Apoptosis assay: Twenty-four hours after irradiation the cells with Δ, V, S-L or L-S dose sequences, they were harvested with trypsin, washed twice with cold PBS and then re-suspended in binding buffer. After that, cells were stained with annexin V-FITC and propidium iodide using the annexin V-FITC apoptosis detection kit I. The FACS flow cytometer was used to quantify the percentage of apoptotic cells.

Statistical analysis: Statistical analysis of data were performed using SPSS 20 software package and are presented as the Mean±SE. One-way ANOVA was used to compare the data among three groups. Student's t-test was used to compare two groups. In all analyses, a p-value less than 0.05 was considered to be statistically significant.

RESULTS

Determination of the threshold dose of HRS: Cell survival measured with the soft agar colony formation assay are presented in Fig. 2. The highest sensitivity observed at 70 cGy which will here after be referred to as D_{hyp}.The corresponding surviving fraction of D_{hyp} will be called SF_{hyp}. The SF_{hyp} in this cell line reached values of 0.72±0.06 at a D_{hyp} of 70 cGy. As the dose was increased further, the SF increased to a value close to 90% at dose of 80 cGy.

Effect of temporal pattern on the cell killing: The ACHN cell line was irradiated with the dose patterns in Fig. 1 to a total dose of 10 Gy over 15 min. Figure 3 shows the measured S_{Δ}, S_{V}, $S_{S\text{-}L}$ and $S_{L\text{-}S}$ after irradiation with or without

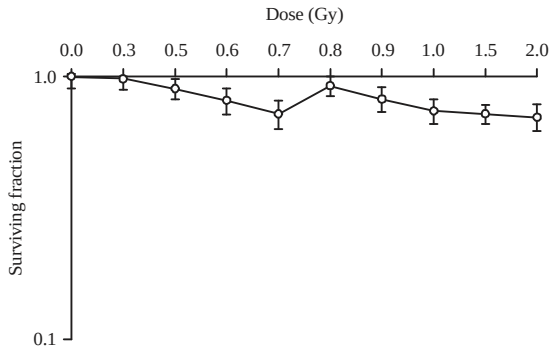

Fig. 2: Survival fraction of human ACHN cell line following irradiation with 0-2 Gy. Hyper-radiosensitivity was appeared at dose of 0.7 Gy

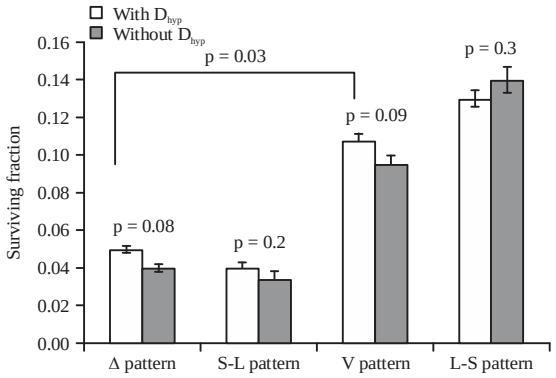

Fig. 3: Comparison of surviving fraction of ACHN cell line after irradiation with Δ, S-L, V or L-S dose patterns in the presence or absence threshold dose of hyper-radiosensitivity at the beginning of pattern using a total dose of 10 Gy and fraction duration 15 min

priming D_{hyp}. There is no significance difference in survival fraction between with and without D_{hyp} at the beginning of all of dose patterns. However, among the different patterns, the survival fraction in Δ and S-L patterns was significantly reduced compared to V and L-S dose sequences. The S_{hyp} of Δ and S-L pattern are 0.05±0.007 and 0.04±0.004, respectively. Without D_{hyp} at the beginning of dose pattern, survival fraction of Δ and S-L pattern is 0.04±0.006 and 0.03±0.008, respectively. No significant difference in the radiosensitivity of the two groups was apparent for V or L-S dose sequence.

Effect of temporal pattern on cell cycle redistribution: Figure 4 shows the cell cycle distribution at 24 h after 10 Gy irradiation using four temporal patterns with or without priming D_{hyp}. In all of the dose patterns, cell cycle distributions at 24 h after irradiation in the presence and absence of priming D_{hyp} showed no significant differences ($p>0.05$).

Effect of temporal pattern on cell apoptosis: To study the effect of the dose patterns with or without priming D_{hyp} on apoptosis of ACHN cells, apoptosis of cells was measured using flow cytometry with the annexin V/PI apoptosis detection kit. Cells were categorized into the following four populations: Early apoptotic (right bottom), late apoptotic (right top) and necrotic (left top) cells. Figure 5 shows that, in all of the dose patterns, there is no significant difference in the percentage of apoptotic cell between with and without priming dose of hyper-radiosensivity groups. In the presence of D_{hyp}, the percentage of apoptosis for Δ, V, S-L and L-S dose sequences were 67.91±5.2, 66.85±4.7, 46.55±3.6 and 41.26±3.9%, respectively.

DISCUSSION

In this study, the threshold dose of HRS for ACHN cell line was determined. Then, effect of four temporal patterns of applied dose (Δ, V, S-L and L-S dose sequence) modeling IMRT with or without a priming dose of HRS were investigated on the cell killing, cell cycle progression and apoptosis of ACHN renal cell carcinoma cell line. The researchers indicated that the cell killing was increased where beams with greatest dose are in the center and beams with smallest dose on the outside (triangle fashion). Similarly, the V-shaped field (where the smallest dose is in center and greatest dose on the outside) decreased the cell killing[8,16,17]. Their studies did not include, however, priming dose of HRS, cell cycle progression and apoptosis.

Although, we have focused on the threshold dose of HRS, there are other variables such as dose rate associated with treatment that could have a potential impact on the effects of the temporal pattern of applied dose. In this study, all fields for all treatments were delivered at a constant dose rate of 3 Gy min^{-1} to the cells. Therefore, it has assumed that dose rate was not a factor in the results of this study.

Earlier studies suggested that HRS is common in radioresistant cell lines[18,19]. To the best of our knowledge, this is the first study that clearly identified the presence of HRS phenomenon in ACHN cell line and finding a low dose hypersensitivity at the 0.7 Gy. Because ACHN cells are radioresistance, they might be considered high HRS prone. It has been suggested that cells remain sensitive at low doses because repair mechanisms are not induced[20].

The result of colony assay showed that S-L and Δ pattern may be most efficient in the presence or absence of D_{hyp} of 0.7 Gy at the beginning of the dose pattern, respectively. Murphy *et al.*[21] suggest that accelerated repair of radiation damaged cells is important mechanism. Accelerated repair is

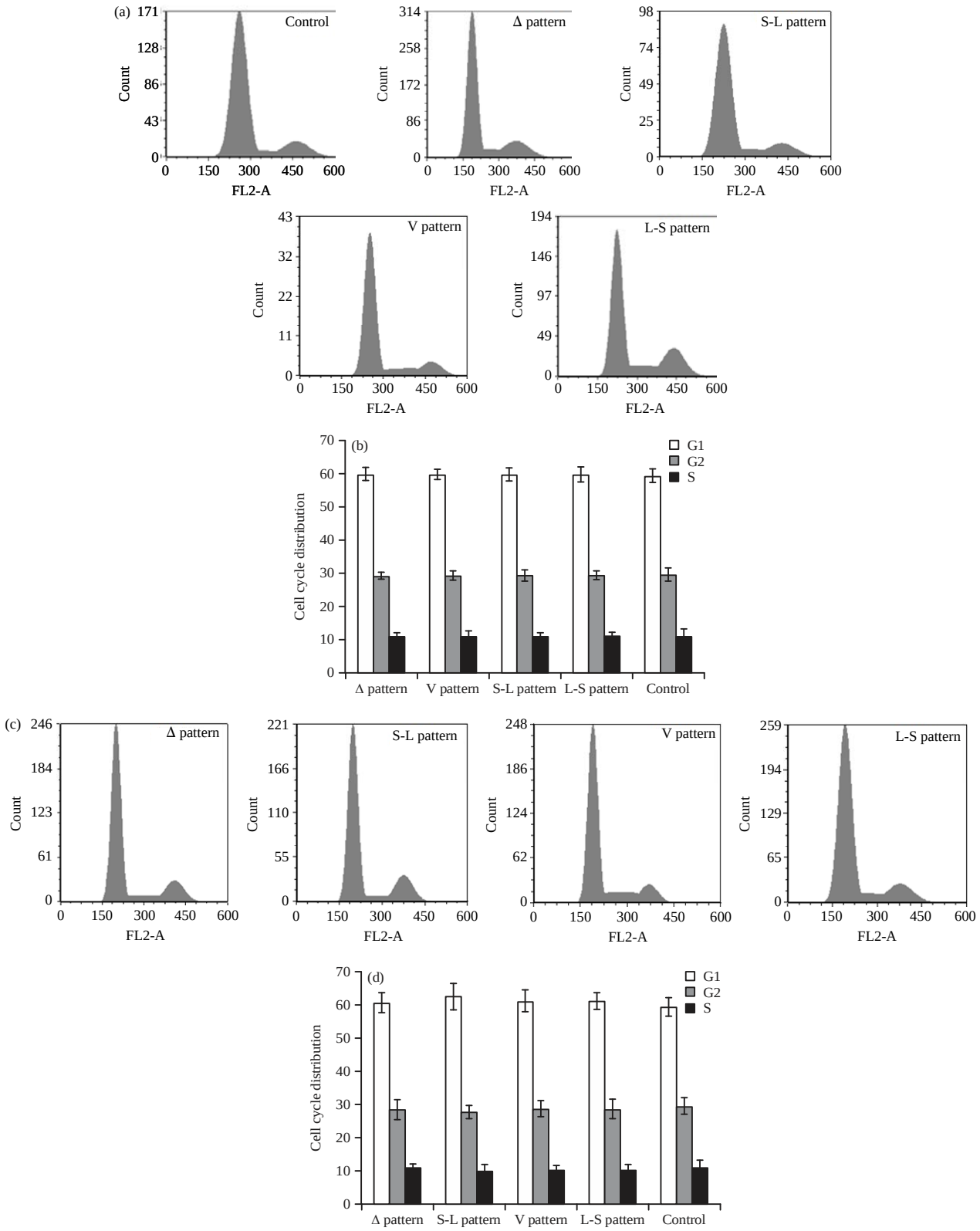

Fig. 4(a-d): Effects of the temporal patterns on cell cycle progression of ACHN cell line 24 h after irradiation (a) With and (c) Without threshold dose of hyper-radiosensitivity at the beginning of dose pattern. Comparison of ACHN cells distribution analyzed by FACS after irradiation using four temporal patterns in (b) Presence and (d) Absence of priming dose of hyper-radiosensitivity

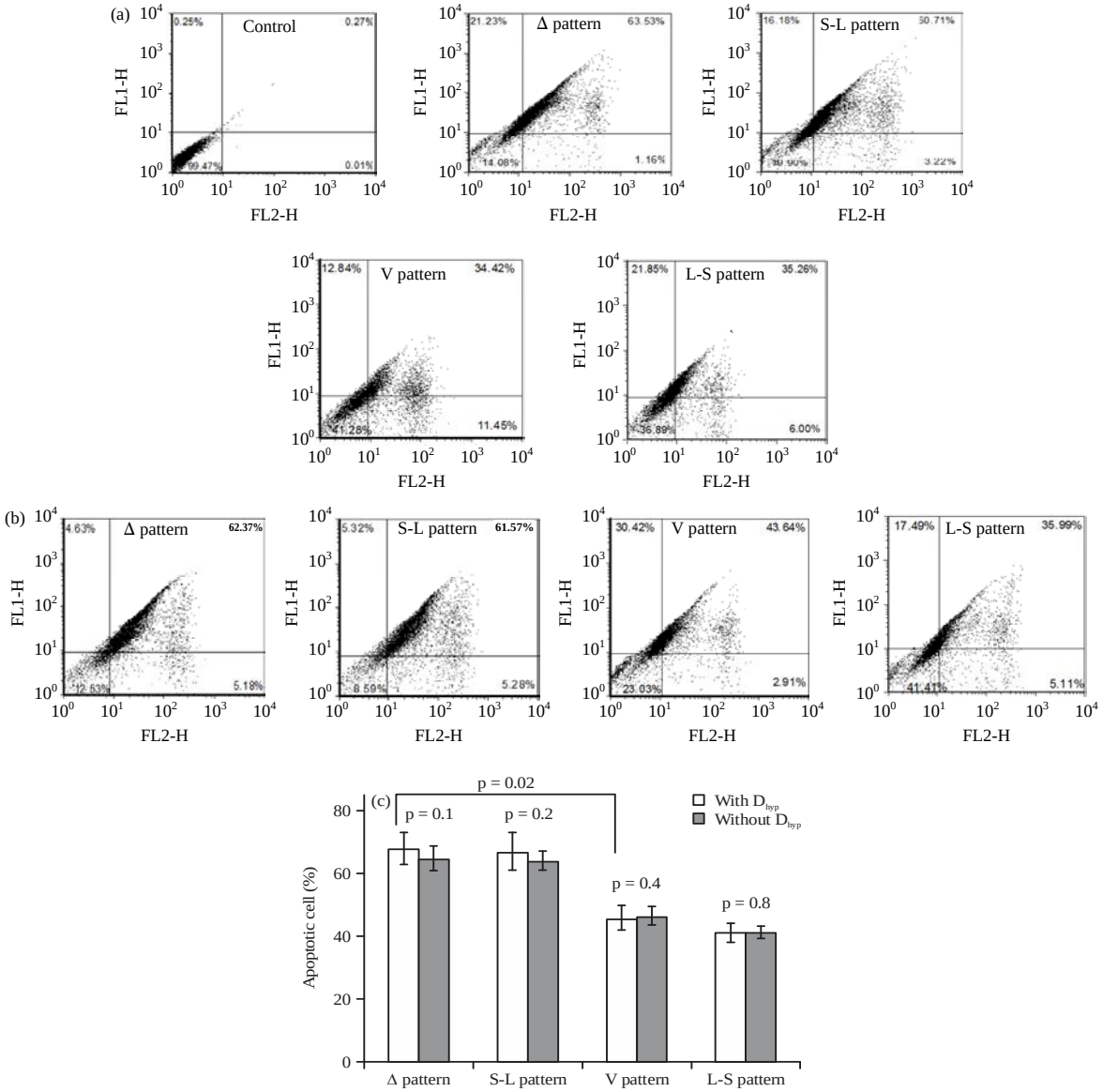

Fig. 5(a-c): Effect of temporal patterns on the apoptosis of ACHN cell line 24 h after irradiation (a) Without and (b) With threshold dose of hyper-radiosensitivity at the beginning of dose pattern and (c) Comparison of the cell apoptosis percentage after irradiation using four temporal patterns in the presence and absence of priming dose of hyper-radiosensitivity

initiated when a relatively high dose is delivered, so that damage from a subsequent dose is rapidly repaired. When a small dose is given first, similar to the dose in this study, this mechanism is not established.

The cell survival percentage of S-L and Δ pattern did not show significant difference when cells irradiated with temporal patterns at the presence or absence of priming dose of hyper-radiosensitivity. It seems that the presence of only single partial fraction with threshold dose of hyper-radiosensitivity at the beginning of the radiation dose pattern could not simulate a mechanisms related to HRS. In a review, Beauchesne[22] showed that HRS could be exploitable in radiotherapy by using very many dose fractions optimally below 1 Gy, an approach it has been termed "Ultrafractionation".

The influence of the temporal patterns of applied dose on cell cycle phase may also have important implications for our understanding of the cellular underlying mechanisms. After irradiation of cells in both dose sequences with and without threshold dose of HRS, there was no apparent changes in the cell populations of the different cell cycle phases. An explanation perhaps is that the patterns of applied dose cannot simulate the cell cycle control system as well as repair mechanism.

To explain the different sensitivity of ACHN cells to radiation patterns, we have investigated programmed cell death or apoptosis. It was found that the apoptotic cells were increased where beams with smallest dose applied at the beginning of dose pattern. The reason for increase of apoptosis in S-L and Δ dose sequence could be due to inactivation of cellular DNA repair mechanisms. These mechanisms are activated when doses at the beginning of V and L-S increase above 1 Gy andled to decrease cell death. Some studies showed that there is a relationship between the enhanced low-dose cell killing and apoptosis in radiation-damaged cells that evade cell cycle arrest. They reported that low-dose radiation exposure causes damage to cells in the G2-phase. These cells evade cell cycle arrest processes and therefore, enter mitosis with unrepaired DNA and die[23,24].

CONCLUSION

New patterns of radiation in IMRT technique was introduced as a most effective tumor cell killing method. Cell cycle control system and apoptosis were checked in our new patterns of radiation delivery to find a molecular mechanism for this effective cell killing. Finding a small priming dose, 0.7 Gy, as a low-dose hyper-radiosensitivity at the beginning of radiation pattern for radio resistant renal cell carcinoma cells could be interesting for the researchers in this field. It is believed that, it is reasonable to take advantage of this enhanced biologic impact by specifying the delivery sequence of treatment fields as a part of the clinical prescription. Ultimately, the temporal manipulation of dose delivery may play a role in treatment optimized to individual patients.

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