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The effect of radiation-induced genotoxic stress on the expression of satellite II and satellite III repeats in breast and colon cancer cells

E. Yilmaz[1,2], E. Ozgur[1], S.B. Gazioglu[3], C.K. Akbas[1], U. Gezer[1], E.E. Yoruker[1*]

[1]Department of Basic Oncology, Institute of Oncology, Istanbul University, 34093 Istanbul, Turkey
[2]Health Sciences Institute, Istanbul University, 34116 Istanbul, Turkey
[3]Department of Immunology, Institute of Experimental Medicine, Istanbul University, 34093 Istanbul, Turkey

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Corresponding author:
Ebru Esin Yoruker, Ph.D.,
E-mail: *akisik@istanbul.edu.tr*

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ABSTRACT

Background: Pericentric human satellite II (HSATII) and III (HSATIII) have been shown to be associated with stress response. Expression status of these satellite repeats has not yet been investigated under radiation-induced genotoxic stress. We evaluated the HSATII and HSATIII expression changes under genotoxic stress in cancer cells. ***Materials and Methods:*** Cell line MCF-7 and cell line HCT-15 were irradiated with 2 and 5 Gy of ionizing radiation, and cell death rates, as a consequence of genotoxic stress, were determined by flow cytometry. The expression of HSATII and HSATIII expressions was assessed by RT-qPCR. ***Results:*** Radiation exposure induced a considerable level of cell death in a dose-dependent manner in both cell types. Compared to untreated cells, HSATII expression declined in MCF-7 cells which were exposed to 2 and 5 Gy radiation, respectively. In HCT-15 cells, the effect of radiation on the HSATII expression was not unified; only higher radiation dose led to a decrease of HSATII expression while 2 Gy increased HSATII expression. The effect of radiation on the HSATIII repeat expression was more pronounced in HCT-15 cells: in MCF-7 cells, HSATIII expression was decreased by 2- and 5-Gy, respectively (p=0.01). In the HCT-15 cells, the rates of HSATIII down-regulation were 3-fold and 2.8 fold by 2- and 5 Gy (p=0.0002 and p=0.02, respectively). ***Conclusion:*** Our findings reveal that genotoxic stress induced by ionizing radiation is associated with a decrease in the expression of pericentric satellites and the expression status of HSATII in these conditions may be dose- and/or cellular context-specific.

INTRODUCTION

Global cancer burden is increasing and expected to rise up to 47% from 2020 to 2040 [1]. The better understanding of tumor development and progression will enable to detect and diagnose cancers in early stages and develop more effective means for treatment and patient management. Besides the genetic alterations, epigenetic mechanisms also contribute to malignant development and tumor progression by affecting tumor suppressor genes and oncogenes [2]. Amazingly, only a small portion of human genome (approx. 2%) encodes proteins whereas a major part of the genome (over than 80%) are transcribed into RNAs not coding for proteins [3]. The recent data indicate that the genes encoding short and long non-coding RNA in the human genome out-number protein-coding genes [4]. The non-coding RNA population of human cells also include RNA molecules from repetitive DNA elements.

Repetitive DNA is defined as the DNA present in multiple copies in the genome and a common feature of eukaryote genomes. The initial sequencing efforts revealed that approximately 55% of the human genome is comprised of repetitive DNA sequences [5]. More recent bioinformatic approaches provided clues that the proportion of repetitive elements in the human genome may be up to two-thirds of the all genome [6]. The main class of repetitive DNA elements are transposable elements which account approximately 45% of genome and primarily composed of retrotransposons. The second class of repetitive DNA constitutes approximately 10% of human genome and includes simple sequence repeats, segmental duplications, tandem repeats and satellite DNA sequences [7].

Centromeric and pericentric satellite sequences, the main constituents of heterochromatin, are implicated in chromosome organization and segregation, kinetochore formation, gene expression as well as heterochromatin regulation [8]. The findings of several previous studies revealed that the expression of centromeric and pericentric satellite sequences increased in cancer tissues compared to the expression in normal tissues [9-11]. In pancreatic cancer, pericentromeric human satellite II (*HSATII*) was the most differentially expressed satellite repeat

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[10,11]. HSATII expression was found to be associated with epithelial-mesenchymal transition and shorter survival of ovarian cancer patients [12]. Furthermore, HSATII has been shown to be expressed in response to DNA damage and induce cell proliferation and migration [13]. These data suggest that the deregulation of HSATII expression may contribute to malignant development. Another member of pericentric satellite repeats is the human satellite III (HSATIII). Several lines of evidence revealed a role of HSATIII in heat-shock stress response [14-17]. The HSATIII-related RNAs were shown to modulate heat-shock-induced transcriptional repression [17]. Expression status of satellite repeats is not yet elucidated under radiation-induced genotoxic stress. Hence, in the present study, we investigated for the first time the RNA expression of HSATII and HSATIII in breast and colon cancer cells which were exposed to ionizing radiation.

MATERIALS AND METHODS

Cell culture

We employed two cell lines as MCF-7, and HCT-15. MCF-7 is *an* epithelial cell line isolated from the tumor of metastatic breast adenocarcinoma and estrogen responsive, and often used to study estrogen receptor positive breast cancers [18]. The MCF-7 cells were purchased from the ATCC (Rockville, MD, USA) and cultured in Dulbecco's modified eagle's medium (DMEM) (Biochrom, Germany), supplemented with 10% fetal bovine serum (Biowest, USA) and 1% glutamine, 2% penicillin-streptomycin (10 U/mL) under standard conditions (37°C and 5% CO_2 humidity). HCT-15 is colon adenocarcinoma cell line and was kindly gifted by Dr. Nihal Karakas (Department of Medical Biology, Medipol University, Turkey). HCT-15 cells were maintained in RPMI 1640 medium (Biochrom) supplemented with 10% fetal bovine serum (Biowest) and 1% glutamine, 2% penicillin-streptomycin (10 U/mL) under standard conditions.

Irradiation of cells

4×10^5 cells were seeded into 60 mm petri dishes and irradiated at total doses of 2 and 5 gray (Gy) 24h after seeding using a Varian DBX Linear Accelerator (Varian Medical Systems Palo Alto CA, USA) at Istanbul University Oncology Institute. Cells were kept under standard growth conditions for further 24h, then harvested using 0.25% Trypsin–EDTA and kept at -80 °C for subsequent analysis.

Cell death detection by flow cytometry

Ionizing radiation as a source of genotoxic stress kills thcells by damaging genomic DNA mainly through the double-strand breaks and may lead to necrotic as well as apoptotic cell death depending on the cell type and dosing [19]. The fraction of dead cells among irradiated cells was evaluated by the Annexin V/PI staining using the Annexin V FITC Apoptosis Detection Kit (Thermo Fisher Scientific, USA) according the manufacturer's instructions. Briefly, cells were resuspended in 100µl of 1X Annexin V binding buffer followed by adding 5µl cold Annexin V -FITC and 1µl of PI (100µg/ml). Cells were incubated for 15min at room temperature (RT) in the dark. After adding 200µl of 1X Annexin V binding buffer cells were examined on a FacsCalibur® flow cytometer (Becton Dickinson Biosciences, San Jose, CA). For the gated cells, the percentages of Annexin V positive (early apoptotic), PI positive (necrotic/dead) and Annexin-V-PI positive (late apoptotic) cells were evaluated. The results were analyzed by FacsCalibur Cell Quest software (Becton Dickinson Biosciences, San Jose, CA).

Expression analysis of HSATII and HSATIII

Total RNA was extracted from the cells using the TriPure RNA Isolation Solution (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's instructions. Briefly, RNA isolation solution was added on cells and incubated at RT for complete disintegration of the nucleoprotein complexes. Following incubation, chloroform incubated at RT for 15 min was added to precipitate nucleic acids. Then, the RNA phase was transferred into fresh tubes and isopropanol was added. The mixture was incubated at RT for 10 min and centrifuged again. RNA-containing pellet was washed with ethanol and air-dried and resuspended in RNAse -free water. After dissolution and quality controls, RNA samples were stored at -80˚C for the cDNA synthesis.

We used differential approach to convert HSATII- and HSATIII-specific RNAs into cDNA. For conversion of HSATII-RN into cDNA, the RevertAid First-Strand cDNA Synthesis kit (Thermo Scientific, USA) was employed using random hexamers. cDNA synthesis for HSATIII was performed as previously described by Valgardsdottir *et al.* 2008 who used a specific primer with the sequence ccgtaaaacgacggccag-ttcccttccattccattattatcc [16]. cDNA synthesis was performed according to the instructions or as described.

RT-qPCR was performed on the LightCycler 480 (Roche, Germany) instrument using SYBR Green (Roche Diagnostics GmbH) as fluorescence dye. PCR conditions were as follows: 40 cycles at 95˚C for 15 sec and 60˚C for 60 sec; 72˚C for 30 sec. GAPDH was used as the internal reference for HSATII and HSATIII. The primer sequences are provided in table 1 with references [16,20,21]. The expression levels were calculated by the $2^{-\Delta\Delta Ct}$ method as previously described [22]. Samples having a Ct > 40 were deemed negative, and melting curve analysis performed after the amplification verified that the correct product had been amplified. The analysis of each sample was performed in triplicate to reduce experimental errors.

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Table 1. The list of Primer sequences used in the study.

Molecule	Primer name	Sequence	Reference
HSATIII	Hur98-R forward M13 reverse	aatcaacccgagtgcaatcg ccgtaaaacgacggccag	16
HSATII	HSATII forward HSATII reverse	catcgaatggaaatgaaaggagtc accattggatgattgcagtcaa	19
GAPDH	GAPDH forward GAPDH reverse	agccacatcgctcagacac gcccaatacgaccaaatcc	20

Statistical analysis

All experiments were performed in triplicates and the mean values were compared for statistical significance. The one-way ANOVA and Student's t-tests were utilized to compare experimental groups. A p value of < 0.05 was considered significant. Statistical analyses were conducted using the GraphPad Prism 5 software.

RESULTS

Determination of cell death rates under radiation-induced stress

Cell death rates were measured 24h after irradiation of cells by flow cytometry (figure 1). We observed a dose-dependent effect of ionizing radiation on cell death rates. In MCF-7 cells, the radiation dose of 2 Gy had a negligible effect while 5 Gy increased the cell death rates by 24%, compared to the levels in the control cells. In HCT-15 cells, the treatment by 2 Gy increased the cell death by 12% and 5 Gy by 34% (figures 1 B and D).

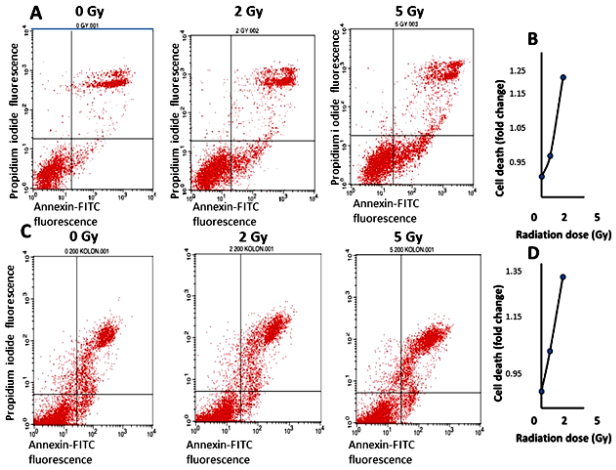

Figure 1. Cell death analysis by flow cytometry. After 24h of seeding, MCF-7 cells and HCT-15 cells were exposed to radiation doses of 0, 2 and 5 Gy and were then further cultured for 24h. Harvested cells were stained with annexin V-FITC and propidium iodide and fluorescence emitted from cells were measured. Propidium iodide (y-axis) was plotted against annexin V-FITC (x-axis). The cells in the bottom left quadrat (Q3) represent viable cells which were stained with neither PI nor FITC; the cells in bottom right quadrat (Q4) are annexin-positive apoptotic cells; Q2 includes late apoptotic cells that were stained with both dyes, and the cells in upper left quadrat (Q1) are necrotic cells which were stained with propidium iodide only. **A.** MCF-7 cells exposed to 0, 2 and 5 Gy radiation, **B.** Total rates of cell death in MCF-7 cells, **C.** HCT-15 cells exposed to 0, 2 and 5 Gy radiation, **D.** Total rates of cell death in HCT-15 cells.

Determination of HSATII and HSATII Expression in Irradiated Cells

Basal expression of HSATII RNA was much higher (333-fold) in MCF-7 cells than in HCT-15. Also HSATIII is expressed in higher levels (15-fold) by MCF-7 cells at baseline than HCT-15 cells. In irradiated MCF-7 cells, we found a substantial decrease in HSATII RNA expression compared to the expression in untreated cells, HSATII RNA expression declined by 5.8- and 9.2-fold in the cells exposed to 2 and 5 Gy radiation, respectively (figure 2A). In HCT-15 cells, the effect of radiation on the HSATII RNA expression was not unified. Compared to the untreated cells, we observed an up-regulation (2.6-fold) of HSATII expression at 2 Gy while its expression declined by 2.3-fold at 5 Gy (figure 2B). These findings suggest that the expression status of HSATII in DNA damage-related stress may be dose- and/or context specific.

The effect of ionizing radiation on the HSATIII repeat expression was consistent; its expression was declined in both cell types with a more pronounced effect in HCT-15 cells. In MCF-7 cells, HSATIII expression was decreased by 1.05 and 1.9-fold by 2 and 5 Gy, respectively ($p=0.01$) (figure 2C). In the HCT-15 cells, the rates of HSATIII reduction were 3- and 2.8 fold by 2 and 5 Gy ($p=0.0002$ and $p=0.02$, respectively; figure 2D).

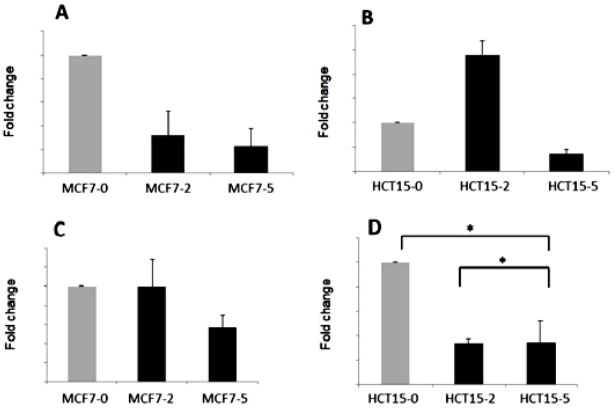

Figure 2. HSATII and HSATIII expression in MCF-7 and HCT-15 cells. Displayed are fold change of HSATII expression in MCF-7 **(A)** and HCT-15 cells **(B)** and fold change of HSATIII expression in MCF-7 **(C)** and HCT-15 cells **(D)**. Bar graphs for fold-change scores represent standard deviations of the mean and where * indicates statistical significance.

DISCUSSION

The available data reveal that the pericentric satellites HSATII and HSATIII are involved in stress response. However, there is no data on their status under radiation-induced genotoxic stress. Therefore, here we examined the HSATII and HSATIII expression in response to genotoxic stress induced by ionizing radiation, with breast cancer cell line MCF-7 and colon cancer cell line HCT-15 as cellular models.

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In our study, we considered the cell death response to DNA damage as a marker of genotoxic stress rather than quantifying the DNA stand breaks numerically by ionizing radiation [23]. As expected, we found a dose-dependent increase in the cell death rates in both cell types. The radiation under used conditions promoted cell death up to 24% in MCF-7 cells and up to 34% in HCT15 cells indicating induced DNA damage response.

HSATII RNA has been shown to be induced in breast cancer cells which were exposed to etoposide or zeocin and increased the HSATII RNA expression correlated with the enhanced migration of cells [13]. In conrast, generally there was a reduction in HSATII RNA expression in breast and colon cancer cells which were exposed to ionizing radiation. Only the low dose (2 Gy) irradiated HCT-15 cells showed a reasonable elevation in the HSATII RNA expression. These findings suggest that the response of HSATII RNA to different DNA-damaging agents may be differential. Similar to the ionizing radiation, etoposide and zeocin have also been shown to induce double-strand breaks [24, 25]. However, a study of Muslimović et al. showed that only 3% of the DNA strand breaks induced by etoposide were double strand breaks and only 10% of etoposide-induced double strand breaks resulted in histone H2AX phosphorylation and toxicity [26]. In contrast, double strand breaks are the main lesions induced by ionizing radiation [27]. The difference in the extent of double strand breaks and related toxicity by chemotherapeutic agents or by ionizing radiation may be a determining circumstance for the expression of pericentric satellites. On the other hand, the differential effect of low dose (2 Gy) and high dose radiation (5 Gy) on HSATII RNA expression in MCF-7 cells suggests that the induction of HSATII RNA by radiation-induced DNA damage may be dose-dependent and cell context-dependent. The mechanisms of such differential effects have to be determined. It is plausible that varying extent of DNA damage by 2 and 5 Gy in MCF-7 cells induce different cellular responses which, in turn affect the HSATII induction.

It is well documented that HSATIII is involved in heat-shock stress response [14-17]. It has also been shown that HSATIII expression is induced by different stress treatments including heavy metals, UV-C, oxidative stress [16]. However, we found a reduction of HSATIII expression upon induction of DNA damage. Similar to HSATTII, also the response of HSATIII to different stress stimuli may be different.

In conclusion, our understanding of radiation induced apoptotic processes responsible for the expression of HSATII and HSAT III is limited and this is the first study to evaluate the expression of pericentric satellites HSATII and HSATIII in genotoxic stress. Unknowns in our understanding of HSATII/ HSATIII repeats' expression in cancer are strongly associated with due to the small number of studies as well as their irregular nature. We did not compare the different DNA-damage inducing agents, however, our findings suggest that the induction of satellite expression by radiation may be differential than by chemotherapeutic agents or other stress sources. We also showed that the expression status of HSATII in these conditions may be dose- and/or cellular context-specific. Further research is needed to explore mechanistic basis of satellite repeat expression in the presence of different genotoxic stimuli.

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***Conflicts of Interests*:** There is no conflict of interest, we declare.

***Ethical Consideration*:** No ethics committee approval was required.

***Author Contribution*:** Concept: EEY, EY.; Design: EEY,EO,EY.; Supervision: EEY. Resources: EEY,EY.; Materials: EO, EEY.; Data collection and processing: EY,EO.; Analysis and interpretation: EO, SBG,EEY.; Literature search: EY, EO, EEY.; Writing Manuscript: EEY,EY.; Critical Review: UG, EEY.

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