RESEARCH ARTICLE

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Effect of Radiotherapy on Activating the Pyroptotic Cell Death Pathway in Breast Cancer Patients: The Role of Serum *GSDMD-CT, NLRP3 and IL-18*

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Abstract

Background: Breast cancer (BC) is the most common form of cancer among women and the second leading cause of cancer-related death worldwide. Several malignancies can be successfully treated with radiation (RT), although radioresistance is still a major contributor to radiotherapy failure. Ionizing radiation (IR) induces pyroptosis in cancer cells. Pyroptosis is a designed method of death connected to routine immunity and directly related to the body ROS content. **Objective for the study:** The aim of this work was to investigate the role of serum *GSDMD-CT*, nucleotide-binding domain and leucine-rich-repeat-containing family pyrin 3 (*NLRP3*) and *IL-18* as predictors of pyroptotic cell death mechanism induced by radiotherapy in breast cancer patients. **Subjects and Methods:** The 70 female participants in this study were divided into two groups: Group (I): 40 breast cancer patients treated with radiotherapy. Group (II): a control group of 30 healthy volunteers with similar ages and sex. Patients with breast cancer received radiation, with a dose of 44 Gray administered over the course of 16 days in five daily fractions of 2.75 Gray each. Two blood samples were taken from breast cancer patients: one before radiotherapy and the other after radiotherapy. While one blood sample was taken from healthy controls. The levels of the circulating pyroptosis biomarkers *IL-18*, *NLRP3*, and *GSDMD-CT* were measured using the ELISA method. **Results:** Our results showed that, there was a significant increase in serum pyroptosis markers *GSDMD-CT*, *NLRP3* and *IL-18* in BC Patients after RT when compared to before radiotherapy.**Conclusion:** Radiotherapy induced pyroptosis in breast cancer patients as a new cell death mechanism. *GSDMD-CT*, *NLRP3* and *IL-18* are biomarkers of pyroptosis that significantly increased post irradiation highlighting enhanced ROS and pyroptosis induction.

Keywords: Breast cancer- Radiotherapy- Pyroptosis- *GSDMD-CT- NLRP3*

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Introduction

The most common malignancy among women worldwide is breast cancer [1]. Breast cancer treatment is multimodal and includes surgery, radiation, chemotherapy, and hormonal therapy. In patients with primary breast cancer following breast conserving surgery and those with locally advanced breast cancer following mastectomy, radiotherapy is used [2].

Radiotherapy is a common modality for tumor treatment in which a therapeutic dose of ionizing radiation (IR) is delivered to the cancer cells. This radiation damages cancerous tissue, with the wanted result being cell death. The dose of radiation is determined by the oncologist, and be contingent on the size, phase and site of the tumor, and the use of any other treatment moods [3]. Radiotherapy may also be used as part of adjuvant treatment when it is assumed after surgery to the area of the tumor bed and local lymph nodes, to kill microscopic cancer cells that may have escaped surgery. Radiotherapy can decrease the hazard of recurrence by 50-66% and is measured critical when breast cancer is treated by breast conservative surgery (breast conservation therapy) [4].

Many cancers are treated with radiotherapy; however radioresistance continues to be a major factor in radiotherapy failure. High-energy ionizing radiation is used in radiotherapy to allow DNA double-strand breaks that lead to cell cycle arrest, senescence, and a number of cell death processes like apoptosis, necrosis, autophagy, and mitotic catastrophe [5, 6]. Developed resistance to apoptosis is a marker of malignancy. Thus, induction of non-apoptotic forms of measured cell death has become

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an emerging anticancer strategy [7].

Another mechanism of programmed cell death is pyroptosis. Inflammatory caspases must start the pore-forming protein gasdermin D (GSDMD) in order for pyroptosis to begin [8]. GSDMD is an executioner of pyroptosis, it is normally in a state of auto-inhibition and can be cleaved by pyroptotic caspases and form the cellular membrane pores [9, 10]. GSDMD holes favor the leak of intracellular components into the extracellular environment. Thus, pyroptosis measured as a lytic, a regulated form of inflammation-induced cell death [11]. Interleukin-1 beta (*IL-1*) and interleukin-18, two pro-inflammatory cytokines, mature as a result of the cleavage of GSDMD by caspase-1 (*IL-18*) [6, 8].

Pyroptosis is strictly connected to many human diseases, including malignancy. Recently, researchers have struggled to associate pyroptosis with numerous tumor behaviors and to treat tumors by regulating pyroptosis and preventing proliferation, invasion and spreading of cancer cells [11]. It was established that ionizing radiation increases the quantity of pyroptosis and raises Caspase-1 activation [12]. Activating tumor (especially apoptosis resistance) pyroptosis causes great beneficial potential for tumor treatment [13]. Therefore in this study aims to investigate the role of serum *GSDMD-CT*, nucleotide-binding domain and leucine-rich-repeat-containing family pyrin 3 (*NLRP3*) and *IL-18* as predictors of pyroptotic cell death mechanism induced by radiotherapy in breast cancer patients.

Materials and Methods

This work included 70 subjects divided into two groups:

Group (I): 40 BC patients were treated with post-operative RT.

Group (II): 30 normal volunteers age and sex matched with BC patients group.

Patients selection

Patients were chosen from among those admitted to cancer management and research department, Medical Research Institute, Alexandria University and Baheya hospital for early detection and treatment of breast cancer, Cairo, Egypt. Prior to their involvement in the study method, all participants were asked to agree to volunteer for the study, and informed written consents were obtained. This study approved by our institution Research Ethics Committee of the Medical Research Institute, Alexandria university (Ethics code: IORG0008812). Cases recruited in the current study were Primary breast cancer patients. No previous history of any other type of cancer or chronic disorders. Metastatic patients at diagnosis and patients received neoadjuvant radiotherapy are excluded from this study.

After diagnosis of breast cancer, patients undergo surgery (either modified radical mastectomy or conservative surgery) after that the tumor's pathological examination included tumor type, grade, tumor size, numbers of axillary lymph nodes involved, and presence or absence of vascular invasion. Evaluations of the expression of Her2/neu, progesterone and estrogen receptors (PR, ER), were also assessed. After surgery, patients received chemotherapy protocol. BC patients were treated with RT with daily irradiation doses of 2.75 Gray (Gy) were provided five days a week for five weeks, yielding a total dosage of 44 Gy.

Controls selection

Controls are healthy Females employees in the same institution, age matched with patients group with normal mammography findings and no previous history of cancer or chronic inflammatory diseases. No history of receiving any radiation therapy.

Blood sampling collection

Two venous blood samples (5ml each) were collected from breast cancer patients: one blood sample was collected before radiotherapy and the second after completing radiotherapy. One- venous blood sample (5ml) was withdrawn from the normal healthy control subjects.

Allow the blood sample to clot for 10-20 minutes at room temperature. Centrifuge at 2000-3000 RPM for 20 minutes. Collect the supernatants carefully. Serum was stored at -80 °C until used. Circulating pyroptosis biomarkers includes *IL-18*, *NLRP3* and *GSDMD-CT* levels were assessed by using ELISA technique according to manufacture instructions (Bioassay Technology Laboratory, England).

Statistical analyses

Data were fed to the computer and analyzed using IBM SPSS software package version 20 (Armonk, NY: IBM Corp). The Shapiro-Wilk test was used to verify the normality of distribution. Quantitative data were described using range (minimum and maximum), median, mean and standard deviation. Student t-test was used to compare between two means of studied groups. Paired t-test was used to compare between two mean periods. Significance of the obtained results was judged at the 5% level of significance ($p<0.05$).

Results

Clinicopathological characteristics of breast cancer patients' group

The general characterizations of breast cancer patients group were represented in Table 1. This table showed that the age of all breast cancer patients ranged from 31 to 72 years. 77.5% of breast cancer patients were of histological grade II, while 15 % were of histological grade III and 7.5% were of histological grade I. Axillary lymph node involvement was pathologically detected in 47.5% of patients, while it was negative in 52.5% of cases. Regarding estrogen and progesterone receptors, estrogen receptors were negative in 5% of patients and positive in 95% of cases, while the progesterone receptors were negative in 7.5% of patients and positive in 92.5% of cases. *Her-2/neu* expression was positive in 37.5% of cases but negative in 62.5%. 87.5% of breast cancer patients were of IDC, while 12.5% were of other types of breast.

Table 1. Clinicopathological Characteristics of Breast Cancer(BC) Patients Group

	Breast cancer patients group (n=40)
Age (Years)	
Mean ± SD	42.52 ± 11.58
Range	(31 – 72)
Histological Grade	
I	3 (7.5 %)
II	31 (77.5 %)
III	6 (15 %)
Axillary lymph node involvement	
Positive	19 (47.5%)
Negative	21 (52.5%)
ER status	
Positive	34 (95%)
Negative	6 (5%)
PR status	
Positive	37 (92.5%)
Negative	3 (7.5%)
Her-2/neu expression	
Positive	15 (37.5%)
Negative	25 (62.5%)
Tumor type	
IDC	35 (87.5%)
Other (ILC & MC and NST)	5 (12.5%)

ER, Estrogen receptor status; PR, progesterone receptor status; Her-2, Human Epidermal growth factor receptor 2; IDC, Invasive Ductal Carcinoma; ILC, Invasive lobular Carcinoma; MC, Mucinous Carcinoma; NST, Invasive Carcinoma of no special type.

Serum NLRP3

The findings are summarized in Table 2 and Figure 1. The mean ± SD levels of serum *NLRP3* were 58.48 ± 17.82 (pg/ml) in BC patients before RT that were increased to 72.18 ± 13.25 (pg/ml) after radiotherapy while it was 48.73 ± 4.49 (pg/ml) in control group. Statistical analysis of these results showed a significant increase in serum NLRP3 in patients with BC after radiation treatment when compared to before radiotherapy and control group (p2<0.001).The difference between BC patients before RT and healthy controls was statistically significant (p1=0.002), and the difference between patients with BC after radiation treatment and healthy controls was statistically significant (p1=0.002).

Serum GSDMD-CT

The findings were summarized in Table 2 and Figure 2. The mean ± SD of serum *GSDMD-CT* was 1.33 ± 0.26 (ng/ml) in BC patients before RT that was increased 1.71 ± 0.41 (ng/ml) after radiotherapy while it was 1.23 ± 0.13 (ng/ml) in control group. Statistical analysis of these findings showed a statistically significant increase in serum *GSDMD-CT* in BC patients after RT when compared to before RT (p2=0.001). The difference between BC patients before RT and healthy controls was not significant (p1=0.061) and the difference between BC patients after RT and healthy controls was statistically significant (p2< 0.001).

Serum IL-18

The findings were summarized in Table 2 and Figure 3. The mean ± SD of *IL-18* was 11.14 ± 1.46 (ng/L) in BC patients before RT that were increased to 13.84 ± 2.44 (ng/L) after radiotherapy while it was 9.39 ± 0.93 (ng/L) in control group. Statistical analysis of these findings showed a significant increased *IL-18* levels in BC patients after RT when compared to before RT (p2=0.001). The difference between BC patients before RT and healthy controls was statistically significant (p1<0.001) and the difference between BC patients after RT and healthy controls was statistically significant (p1< 0.001).

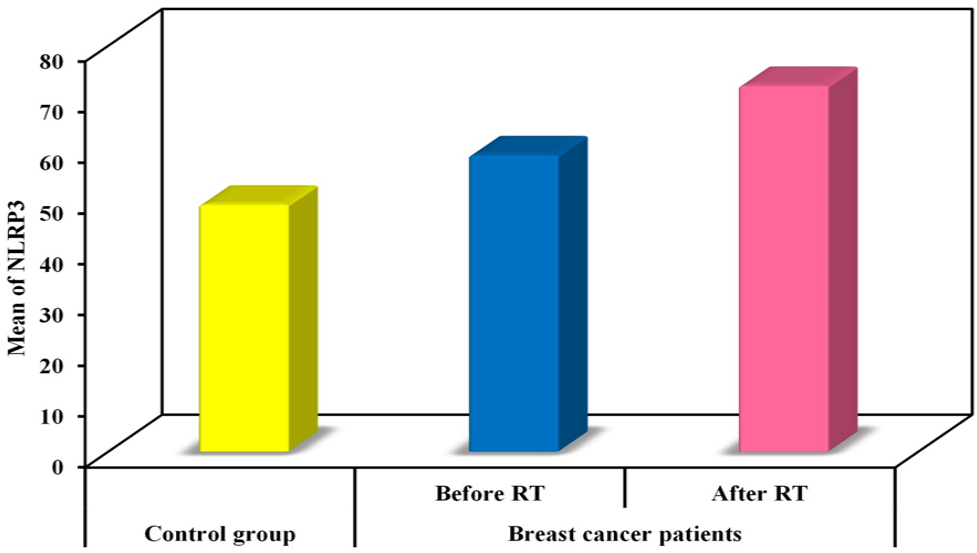

Figure 1. Bar Chart Showing Levels of Serum *NLRP3* in All Studied Groups

Table 2. Serum ₚevels of *NLRP3, GSDMD-CT* and *IL-18* in Breast Cancer Patients before and after RT Treatment in Comparison to Control Group

NLRP3 (pg/ml)	Control group	Breast cancer patients (n=40)	
	(n=30)	Before RT	After RT
Mean ± SD.	48.73 ± 4.49	58.48 ± 17.82	72.18 ± 13.25
p1		0.002*	0.001*
p2		0.001*	
GSDMD-CT (ng/ml)			
Mean ± SD.	1.23 ± 0.13	1.33 ± 0.26	
			1.71 ± 0.41
p1		0.061	0.001*
p2		0.001*	
IL-18 (ng/L)			
	9.39 ± 0.93	11.14 ± 1.46	13.84 ± 2.44
Mean ± SD.			
p1		0.001*	0.001*
p2		0.001*	

p1, p value for comparing between before and after RT with control group; p2, p value for comparing between before and after radiotherapy treatment; *, Statistically significant at $p \leq 0.05$.

Discussion

The way of cell death can be roughly divided into two categories: cell necrosis and PCD (programmed cell death). Pyroptosis is a kind of apoptosis, its occurrence depends on the gasdermin protein family and it will produce inflammatory response [14]. In the present study, serum level of *NLRP3* was higher in patients than in the control group, after radiotherapy, statistically significant increased serum *NLRP3* was observed in patients versus pre radiotherapy treatment indicating regulation of the secretion and activation of inflammatory cytokines and promoting pyroptosis.

As NLRP3 activation causes pyroptotic, immunogenic cell death and the release of pro-inflammatory factors, direct inflammasome activation within the tumor may be an important mechanism to engage antitumor immunity. The *NLRP3* inflammasome activation, *IL-1β* production, and pyroptosis were downregulated by knockout of *NLRP3* [15]. These results suggest that radiation induces NLRP3 inflammasome activation and pyroptosis in breast cancer cells. Zhang et al. [16] reported that *NLRP3* inflammasome activation mediates radiation-induced pyroptosis in breast cancer patients. Previous study suggested that IR not only was related to immune system reaction such as promoting proinfammation factors expression, which was also involved in necrosis, but also had close relationship with cell death, such as apoptosis. Given the fact that pyroptosis possesses the characteristics of both apoptosis and necrosis [15].

Pyroptosis, defined as Caspase-1-dependent programmed and pro-inflammatory cell death, is distinct from any other programmed cell death and results in cell lysis and pro-inflammatory cytokine release [17]. As pyroptosis occurs after activation of caspase-1 and many studies that have focused on the inflammatory

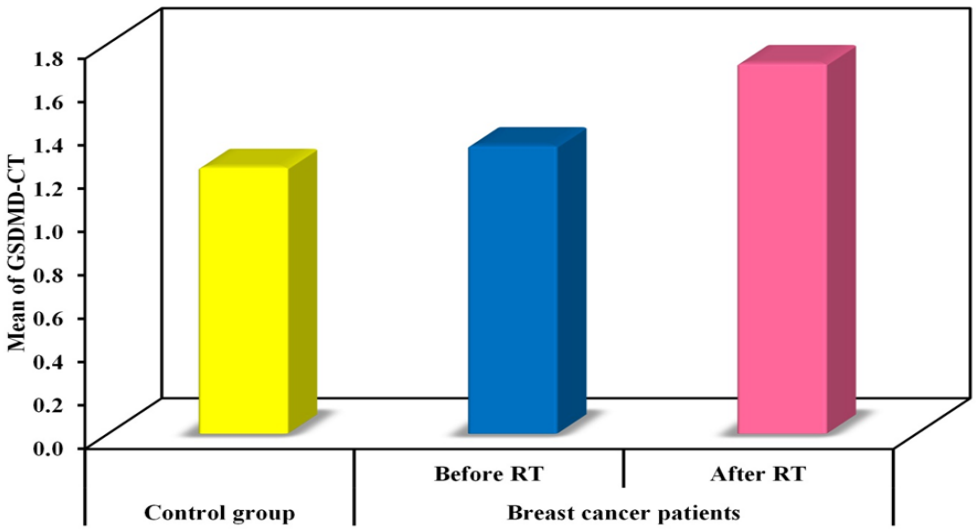

Figure 2. Bar Chart Showing levels of serum *GSDMD-CT* in All Studied Groups

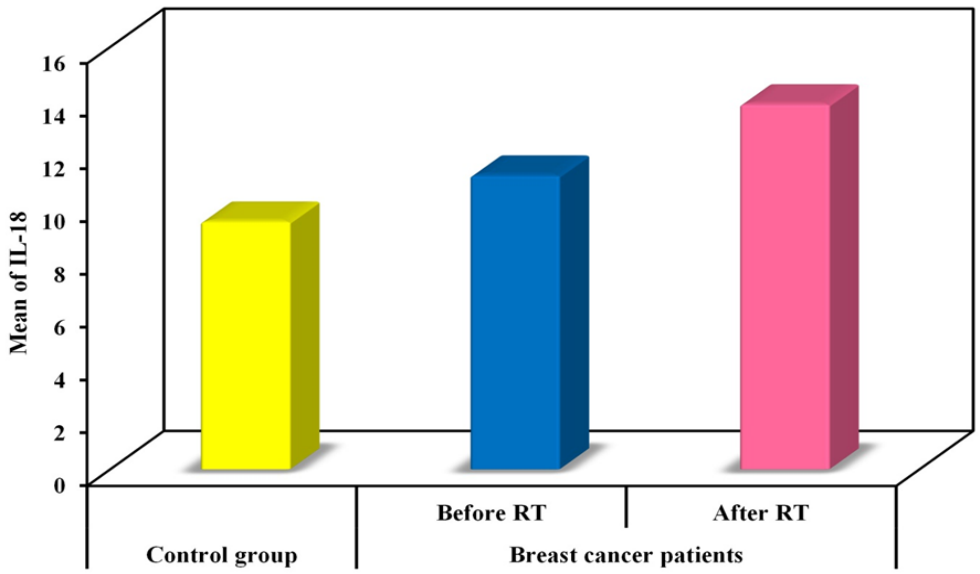

Figure 3. Bar Chart Showing Levels of *IL-18* in All Studied Groups

responses under radiation detected controversial results about the links between radiation exposure and *NLRP3* inflammasome activation, author then tested the role of the *NLRP3* inflammasome in radiation and in the pyroptosis induced by radiation in breast cancer cells using ELISA techniques. Interestingly, the results imply that the cells exposed to radiation shows evidence of dose-dependent Caspase-1 activation and *IL-1β* production and that the Caspase-1 activation and *IL-1β* production is dependent on *NLRP3*, since *NLRP3*knock out diminishes the quantity of cleaved-Caspase-1 (p10) and IL-1β. More vitally, *NLRP3* knock out can also significantly lessen the proportion of pyroptosis induced by radiation [12, 18].

Accordingly, we believe that radiation can induce pyroptosis through activating the *NLRP3* inflammasome. This finding is novel and further supports the idea that *NLRP3*-Caspase-1 inflammasome activation is essential in radiation induced cell and tissue damage. The activation of the *NLRP3* inflammasome is generally believed to require two signals, signal 1 being Toll-like receptor activation leading to cellular priming and upregulation of *NLRP3* expression and signal 2 is being an additional stimulation of these cells with damage associated molecular patterns (DAMPs). Indeed, the direct effects of radiation, such as damage to lysosomes, nuclear DNA or mitochondrial DNA, could be the signal 2 of *NLRP3* inflammasome activation. In addition, different tissue or cell types may react differently to ionized radiation [12].

In the present study, serum GSDMD and *IL-18* and statistically and significantly increased after radiotherapy treatment yet it did not achieve the same level as the control group. This goes in line with Liu et al. [12] who found that the activity of caspase-1 and GSDMD were enhanced, the secretion of *IL-1β* was increased, and showed pore-formation activity, which suggesting the occurrence of pyroptosis. These findings direct us to improve the efficacy of radiotherapy by regulating pyroptotic triggering.

Ionizing radiation activates the process of pyroptosis, which leads to the activation of Caspases, shearing of GSDMD, and the release of inflammatory factors resulting in cascade amplification. Mature Caspase-1 promotes the development of IL-1β, *IL-18* and other inflammatory cytokines and shears GSDMD, whose N-terminal domains aggregate and form large oligomeric pores in the cell membrane, which triggers the release of inflammatory cytokines and leads to death of cells [19].

Wang et al. [20] study which conducted to elucidate the mechanisms of GSDMD-facilitated pyroptosis proved that, through disturbing pyroptotic cell death by GSDMD inactivation, the expression of *IL-1β* and *IL-18* was successfully decreased in plasma, proposing that GSDMD pore formation participated in proinflammatory cytokines release from immune cells. It is important to highlight that pyroptotic death is an inflammatory form of programmed cell death characterized by cellular swelling and rupture, lysis, nuclear condensation, DNA fragmentation and *IL-1β* and *IL-18* leakage, exacerbating the inflammatory response in the extracellular space. Pyroptosis induces DAMPs, such as HMGB1, IL-1α, and adenosine-triphosphate (ATP), release and, hence, promotes a local immune response. These molecules are involved in many types of cancer and contribute to the tumorigenic potential of inflammosome activation. Pyroptosis-induced products can also limit the survival of tumor cells, and trigger, through immunogenic signals, the activation of the innate immune response blunting cancer progression [20].

Finally, pyroptosis is a new cancer treatment target. yet the mechanisms that regulate it are complex and need to be deeply explored notably as regards its inducers or its inhibitors. Reducing cancer capacity to evade cell death by pyroptosis is a potential therapeutic strategy especially for tumor resistant to apoptosis.

In conclusion, Radiotherapy induced pyroptosis in breast cancer patients as a new cell death mechanism. *GSDMD-CT*, *NLRP3* and *IL-18* are biomarkers of pyroptosis that significantly increased post irradiation highlighting enhanced ROS and pyroptosis induction.

Author Contribution Statement

Taha I. Hewala : Designing & writing the manuscript. Sanaa A. El-Benhawy: Research proposal idea, literature review, writing the manuscript & practical lab part of the paper. Yasmine N. Elwany: Helped in sample collection, following up the cancer patients radiotherapy participated in manuscript writing & paper submission. Hossam El masry: Participated in samples collection. Ayman Elrhawy: Contributed in data analysis, practical part, & participated in manuscript writing.

Acknowledgements

All authors have made significant contributions to this work.

Ethical Declaration

Written informed consent was obtained from all study subjects. Also, approval of the ethics committee of the Medical Research insitute (Ethics code IORG : 0008812) Alexandria University , Egypt , was obtained prior of the study. All procedures performed in our study were in accordance with ethical standards of our institution & with the 1975 Helsiniki declaration as revised in 2008.

Study Registration

Was the study registered in any registering dataset: NO (for clinical trials, guideline, meta analysis)

Recommendations

From the above findings, we may recommend the following:

Pyroptosis could be targeted in the therapy of BC patients.

Conflict of Interest

No conflict of interest is declared.

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