

RESEARCH ARTICLE

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miR-31 Expression Correlates with Radiation-Induced Bystander Responses in Breast Cancer Cells

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Abstract

Purpose: This study investigated the association of *miR-31* expression with radiation-induced bystander responses in non-irradiated MDA-MB-231 cells exposed to radiation-conditioned medium derived from irradiated MCF-7 breast cancer cells. **Methods:** MCF-7 cells were exposed to 0.5, 1, 2, and 4 Gy of 6 MV X-rays. Conditioned media were collected at 0, 24, and 48 hours' post-irradiation and applied to MDA-MB-231 cells. Cell viability was assessed via MTT assay. Apoptosis and cell cycle behaviors were assessed using flow cytometry. Wound healing assays were conducted to assess migration, and RT-qPCR was used to quantify *miR-31* expression. **Results:** MDA-MB-231 cells underwent dose- and time-dependent alterations associated with conditioned media from irradiated MCF-7 cells. Apoptosis increased significantly over time, especially in the 2 and 4 Gy groups. Cell cycle analysis showed shifts in the G1 and S phases. Migration rates were enhanced at specific time points, particularly in the 0.5 and 2 Gy groups. The expression of *miR-31* was significantly increased in treated MDA-MB-231 cells. **Conclusion:** Our findings suggest a potential association between altered *miR-31* expression and radiation-induced bystander responses in breast cancer cells. Further mechanistic studies are required to clarify its functional role and therapeutic relevance.

Keywords: breast cancer, radiotherapy, *miR-31*, bystander, MDA-MB-231 and MCF-7 cell lines

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Introduction

Breast cancer remains one of the most frequently diagnosed malignancies and a leading cause of cancer-related mortality among women worldwide [1]. Therapeutic resistance and recurrence remain complex clinical problems, even with significant progress in early identification and multimodal therapies involving surgery, chemotherapy, hormone therapy, and radiation. The variety of breast cancers combined with their ability to adapt and avoid cytotoxic effects calls for a better knowledge of both the direct and indirect cellular reactions to treatment, most importantly, radiation [2, 3].

An essential component of the treatment for breast cancer, ionizing radiation (IR) is well-known for causing DNA damage and inducing cell death in malignant cells via direct interactions. Still, an increasing number of studies have shown a variety of non-targeted effects (NTEs) resulting from radiation [4, 5]. Of them, the bystander effect (BE) has attracted the most interest. BEs are biological reactions observed in cells that are not directly irradiated but rather exposed to signals from neighboring cells that have been irradiated. Together, these impacts

affect treatment results, including chromosomal instability, changed gene expression, apoptosis, proliferation, and changes in cellular behavior [6, 7].

The transmission of bystander signals is believed to be associated with several factors, including cytokines, reactive oxygen species (ROS), and, most notably, extracellular vesicles (EVs) such as exosomes [8, 9]. Exosomes are nano-sized membrane-bound vesicles (typically 30–150 nm) that are secreted by most cell types and play critical roles in intercellular communication. They may move a variety of products, including proteins, lipids, messenger RNAs (mRNAs), and microRNAs (miRNAs), to destination cells, therefore altering different biological processes [8, 10]. Recent studies have demonstrated how exosomes have been suggested to facilitate the response of tumor cells to radiation and enhance the spread of radiation-induced bystander signals. It also suggests that exosomes can be active participants in therapeutic resistance or sensitivity and can serve as biomarkers [11, 12].

Among the exosome-enclosed regulators, microRNA-31 (*miR-31*) has emerged as a key molecule in cancer biology. Small non-coding RNAs called miRNAs

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attach to the 3'-untranslated region (3'-UTR) of target mRNAs to control gene expression post-transcriptionally, so either degradation or translational repression results. Depending on the type of cancer, stage, and microenvironmental setting, *miR-31* appears to have dual action, functioning either as an oncogene or a tumor suppressor [13]. In breast cancer, *miR-31* expression is significantly higher in non-metastatic MCF-7 cells and nearly absent in metastatic MDA-MB-231 cells, suggesting a potential role in suppressing invasiveness and metastasis [14]. Furthermore, studies have reported that *miR-31* can modulate radiosensitivity by influencing apoptosis, cell cycle progression, and epithelial-mesenchymal transition (EMT) pathways [15].

Despite these findings, the potential involvement of *miR-31* in radiation-induced bystander effects, particularly in the context of extracellular vesicle-associated communication, remains poorly understood. Limited research has explored whether *miR-31* expression released from irradiated tumor cells can alter the biological behavior of neighboring, non-irradiated cells. This method not only clarifies RIBEs but also identifies new molecular targets to overcome resistance and enhance the effectiveness of radiation [16, 17].

Given the complexity of radiation-induced effects and the emerging relevance of extracellular vesicle-associated signaling in tumor biology, further investigation is warranted to determine how *miR-31* expression influences cellular processes, such as proliferation, apoptosis, migration, and cell cycle regulation, in neighboring cancer cells. Elucidating this connection may provide novel strategies for improving the effectiveness of radiation and minimizing off-target adverse effects in breast cancer management [18, 19].

Therefore, this study aimed to investigate whether radiation-conditioned medium derived from irradiated MCF-7 breast cancer cells is associated with altered *miR-31* expression and bystander responses in non-irradiated MDA-MB-231 cells.

Materials and Methods

Cell Lines and Culture Conditions

This study utilized two human breast cancer cell lines representing distinct molecular subtypes: MCF-7, a luminal A, hormone receptor-positive, non-metastatic cell line, and MDA-MB-231, a triple-negative, basal-like, metastatic cell line. The rationale for using both was to evaluate whether radiation-induced bystander effects (RIBEs), particularly those potentially associated with *miR-31*-related signaling, would differ based on the molecular phenotype and intrinsic radiosensitivity of the cells. MCF-7 cells are more radiosensitive than MDA-MB-231 cells, which display greater radioresistance, aggressive migration, and altered cell cycle behavior. Both cell lines were obtained from the Iranian Biological Resource Center and cultured in Dulbecco's Modified Eagle's Medium (DMEM; BIO-IDEA, Iran), improved with 1% penicillin/streptomycin and 10% fetal bovine serum (FBS). They were humidified in a 37°C incubator with 5% CO₂. Between passages 3 and 5, all tests were

conducted using logarithmic growth phase cells.

Irradiation Protocol

MCF-7 cells were exposed to 6 MV X-ray photon beams using a Varian 2100C/D linear accelerator (Varian Medical Systems, USA). The irradiation dose rate was 200 cGy/min (2 Gy/min). Cells were seeded into T-25 flasks, allowed to adhere for 24 hours, and positioned on a 1.5 cm solid water-equivalent plexiglass phantom to ensure backscatter equivalence and build-up effect. Irradiation was supplied to ensure total coverage of the flask surface; the source-to-surface distance (SSD) was 100 cm. Radiation doses administered were 0.5, 1, 2, and 4 Gy. Non-irradiated MCF-7 cells served as the sham control group.

Conditioned Medium Collection and Treatment

Following irradiation, MCF-7 cells were incubated for 0, 24, and 48 hours. Subsequently, the conditioned medium (CMs) was collected, centrifuged at 1500×g for 10 minutes, and cleared of cells and debris through a 0.22 µm filter. For storage, CMs were maintained at -80°C. MDA-MB-231 cells were planted in 6-well plates, allowed to adhere overnight, and then exposed to medium of irradiated MCF-7 cells that had been treated with 0.5, 1, 2, or 4 Gy of X-rays. They were then examined for radiation-induced bystander effects 24 hours later. Radiation-Conditioned Media (RCM) was the exposure condition; throughout the study, the treated cells were identified as RCM-exposed MDA-MB-231 cells, and two control groups were included:

NC (Negative Control): Untreated MDA-MB-231 cells.

PC (Positive Control): MDA-MB-231 cells treated with CM from non-irradiated MCF-7 cells.

Cell Viability

The MTT method (Sigma-Aldrich, USA) was used to evaluate cell viability. Each well contained 1.5×10^4 MDA-MB-231 cells overall from 96-well plates. 10 µL of MTT solution (5 mg/mL) was applied per well and incubated for 4 hours at 37°C after treatment with CMs for 24 hours. Subsequently, the supernatant was removed, DMSO was added to break down the formazan crystals, 100 µL, and the absorbance was calculated at 570 nm employing an ELx808 microplate reader (BioTek, USA). Every measurement was done three times.

Apoptosis Assay

Following the manufacturer's instructions, an Annexin V-FITC/PI staining kit (eBioscience, USA) was used to quantify apoptosis. After 24 hours of CM exposure, MDA-MB-231 cells were trypsinized, washed with cold PBS, and resuspended in a binding buffer. Cells were incubated with Annexin V-FITC (5 µL) and PI (5 µL) for 15 minutes in the dark. Flow cytometry was carried out using a BD FACSCalibur flow cytometer, and the FlowJo program was utilized to analyze at least 100,000 events per sample.

Cell Cycle Analysis

After treatment with RNase A (100 µg/ml), cells were stained with PI (50 µg/ml) for 30 minutes at room temperature. Data were collected using flow cytometry, and FlowJo software was used to analyze the proportion of cells in the G₀/G₁, S, and G₂/M phases. Generating representative histograms helped to verify appropriate gating and peak separation.

Wound Healing Assay

A scratch investigation was conducted to evaluate cell migration. MDA-MB-231 cells were implanted in six-well plates and allowed to grow to 90% confluence. A sterile 200 µl pipette tip was used to create a straight scratch. After removing debris with PBS, cells were treated with CMs and incubated at 37°C. An Olympus phase-contrast microscope was used to capture images of wound closure at 0, 24, and 48 hours, and the scratch area was quantified using ImageJ software. Migration was calculated using the formula:

$$\text{migration rate (\%)} = \frac{A_{0h} - A_t}{A_{0h}} \times 100$$

RNA Extraction and Quantitative Real-Time PCR (qRT-PCR)

Total RNA was extracted using the RNX-Plus reagent (SinaClon, Iran), and RNA purity and concentration were verified using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, USA). Samples with an A260/A280 ratio between 1.8 and 2.0 were used for further analysis. Primers were designed based on mature miRNA sequences obtained from the miRBase database and were evaluated for specificity, melting temperature compatibility, and absence of secondary structures or primer-dimer formation using Primer-BLAST analysis (Table 1). Complementary DNA (cDNA) was synthesized using the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, USA) with specific stem-loop primers for *miR-31* and *Snord-48*. *Snord-48* was selected as the internal reference gene due to its reported stable expression across different experimental conditions and breast cancer cell lines, making it suitable for normalization of miRNA expression data [20]. qPCR was performed using SYBR Green PCR Master Mix (Yekta Tajhiz Azma, Iran) on an ABI StepOnePlus Real-Time PCR System. Each reaction was run in triplicate. PCR efficiency for each primer set was confirmed to be between 90% and 110% by standard curve analysis. Relative expression levels were calculated using the $2^{-\Delta\Delta C_t}$ method.

Statistical Analysis

Statistical analyses were performed using GraphPad Prism 8 software (GraphPad Software Inc., USA). Data are presented as mean ± standard deviation (SD) from at least three independent experiments. Comparisons among multiple groups were conducted using two-way analysis of variance (ANOVA) followed by Tukey's post hoc multiple comparison test. Differences were considered statistically significant when $p < 0.05$.

Results

A dose-dependent reduction in MDA-MB-231 cell viability at 24 hours results after RCM exposure

MDA-MB-231 cells were evaluated for bystander cytotoxic effects after exposure to RCM. Compared to both the NC and PC, the MTT test at 24 hours' post-exposure demonstrated a moderate but statistically significant decrease in cell viability in the 2 Gy and 4 Gy groups, respectively. Although the degree of difference did not surpass 4%, the decrease was nevertheless noteworthy when compared to the 0.5 Gy group ($P < 0.05$). These results suggest that higher-dose RCM can have a detectable but mild inhibition on metabolic activity in bystander MDA-MB-231 cells (Figure 1).

RCM triggers progressive apoptotic signaling in MDA-MB-231 cells, with maximum effect at 48 hours

According to flow cytometry analysis of Annexin V-FITC/PI-stained MDA-MB-231 cells, RCM significantly increased death in a dose- and time-dependent manner. Except for a slight decrease in the 0.5 Gy RCM group, no significant differences were observed among most groups at 0 h. While the 0.5 Gy and 1 Gy groups remained similar to controls, after 24 h, apoptotic rates notably increased in the 2 Gy and 4 Gy RCM groups relative to both the NC and PC groups ($P < 0.05$ to $P < 0.0001$). A significant increase in death was seen at 48 h in both the 2 Gy and 4 Gy groups; the 4 Gy RCM group had the highest death percentage ($P < 0.0001$). Time-dependent comparisons within each dosage group revealed that, especially in the 0.5 Gy, 2 Gy, and 4 Gy groups, extended exposure (48 h) resulted in a gradual rise in dead cells. These findings show that, in MDA-MB-231 cells, RCM obtained from higher radiation doses has a greater bystander pro-apoptotic impact, which becomes more pronounced with prolonged incubation durations (Figure 2).

RCM exposure induces S-phase or G1 arrest in MDA-MB-231 cells depending on radiation dose and time

Various variations in cell cycle distribution were found by flow cytometric measurement of DNA content upon RCM exposure. Early replicative stress was observed in MDA-MB-231 cells following 0.5 Gy RCM, leading to a significant accumulation of cells in the S phase compared to the NC and PC groups at 0 h, $P < 0.0001$. In lower dosage groups, no significant alterations were observed within 24 hours; however, 4 Gy treatment resulted in considerable G1 phase enrichment ($P < 0.01$). Effective G1 phase arrest and a corresponding decrease in S-phase population ($P < 0.0001$) were observed in both the 2 Gy and 4 Gy groups after 48 h, indicating increasing inhibition of DNA synthesis. These results demonstrate that RCM affects the cell cycle in a dose- and time-dependent manner; low-dose RCM induces S-phase arrest, whereas higher doses cause G1 arrest (Figure 3).

RCM exposure enhances MDA-MB-231 cell migration at low and intermediate radiation doses

The effect of radiation-conditioned medium (RCM) on the migratory behavior of MDA-MB-231 cells was

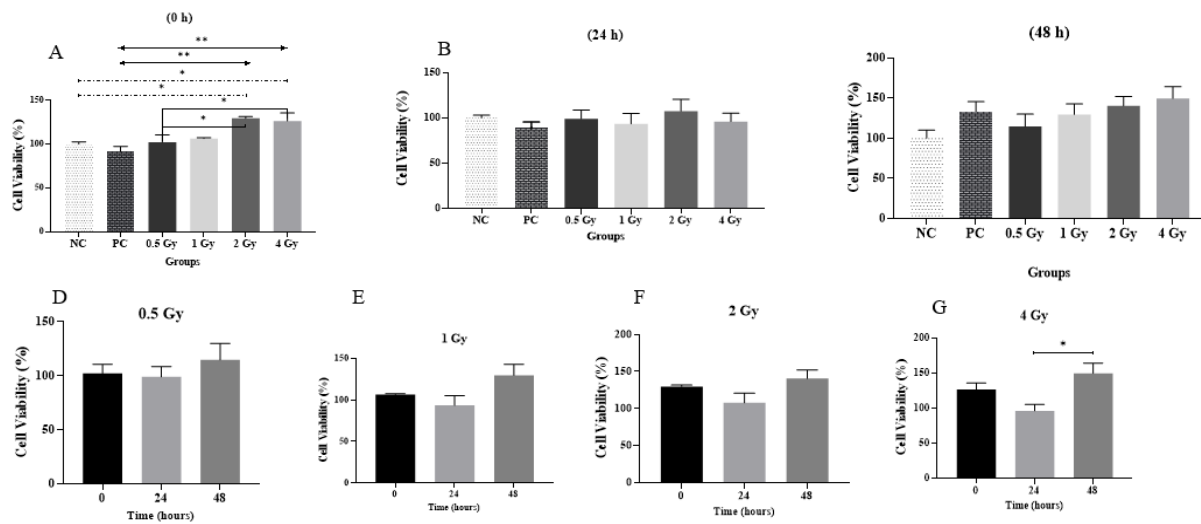


Figure 1. Effects of Radiation-Conditioned Medium (RCM) on MDA-MB-231 Cell Viability. (A–C) Cell viability following exposure to RCM collected from MCF-7 cells irradiated with 0.5, 1, 2, and 4 Gy at 0 h (A), 24 h (B), and 48 h (C). (D–G) Time-dependent changes in viability in MDA-MB-231 cells exposed to RCM derived from 0.5 Gy (D), 1 Gy (E), 2 Gy (F), and 4 Gy (G) irradiated MCF-7 cells. Cell viability was assessed using the MTT assay. Data are presented as mean $\pm$ SD from three independent experiments. Statistical analysis was performed using two-way ANOVA followed by Tukey's post hoc test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

investigated using wound healing assays. In cells exposed to RCM generated from cultures irradiated with 0.5 Gy and 2 Gy, results revealed a significant time- and dose-dependent increase in migration. Notably, the 2 Gy group showed a clear peak at 24 h ($P < 0.01$), while the 0.5 Gy group exhibited improved wound closure at both 24 h and 48 h in comparison to the beginning ($P < 0.01$). By comparison, 4 Gy RCM-treated cells showed no appreciable increase in migration above the control groups. These results suggest that lower radiation levels

trigger bystander- associated signals that may promote migration activity (Figure 4).

miR-31 expression is significantly upregulated in response to low and moderate-dose RCM exposure

Significant changes in *miR-31* expression in RCM-exposed MDA-MB-231 cells were shown by quantitative real-time PCR analysis. Expression levels in cells treated with RCM from cultures irradiated with 0.5, 1, and 2 Gy were significantly higher at 0 hours than

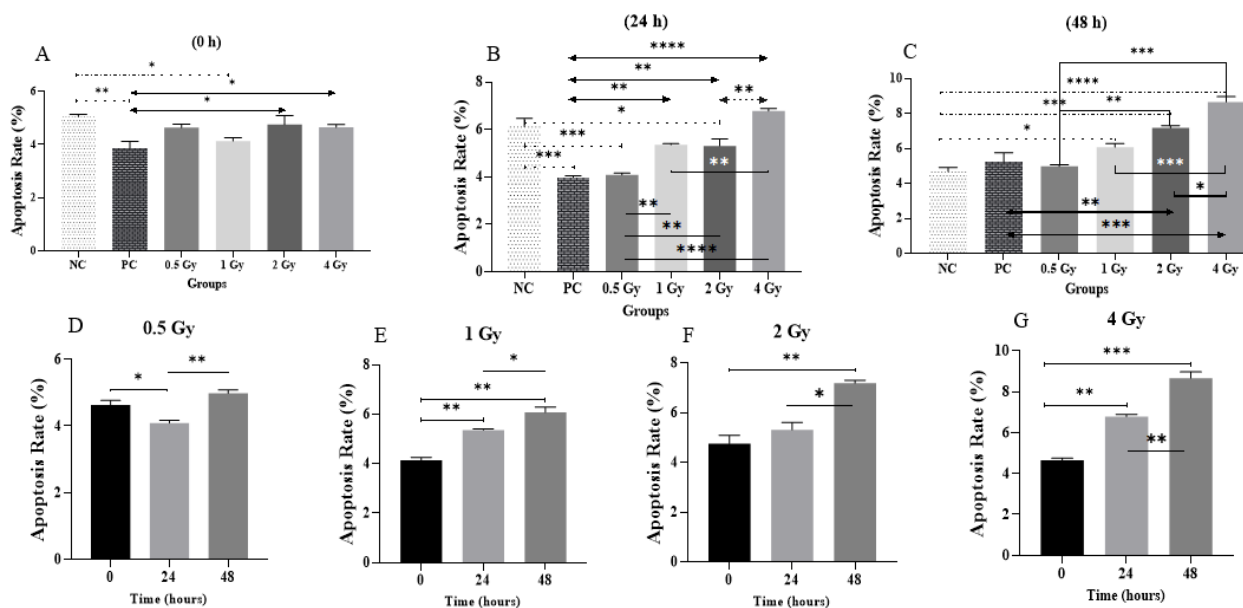


Figure 2. RCM Gathered from Irradiated Cultures Induced Apoptosis in MDA-MB-231 Cells. (A–C) Comparison of apoptotic rates among different RCM dose groups at 0 h (A), 24 h (B), and 48 h (C). (D–G) Time-course apoptotic changes within each RCM dose group: 0.5 Gy (D), 1 Gy (E), 2 Gy (F), and 4 Gy (G). Apoptosis was assessed using Annexin V-FITC/PI staining followed by flow cytometry. Data are presented as mean $\pm$ SD from three independent experiments. Statistical analysis was performed using two-way ANOVA followed by Tukey's post hoc test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

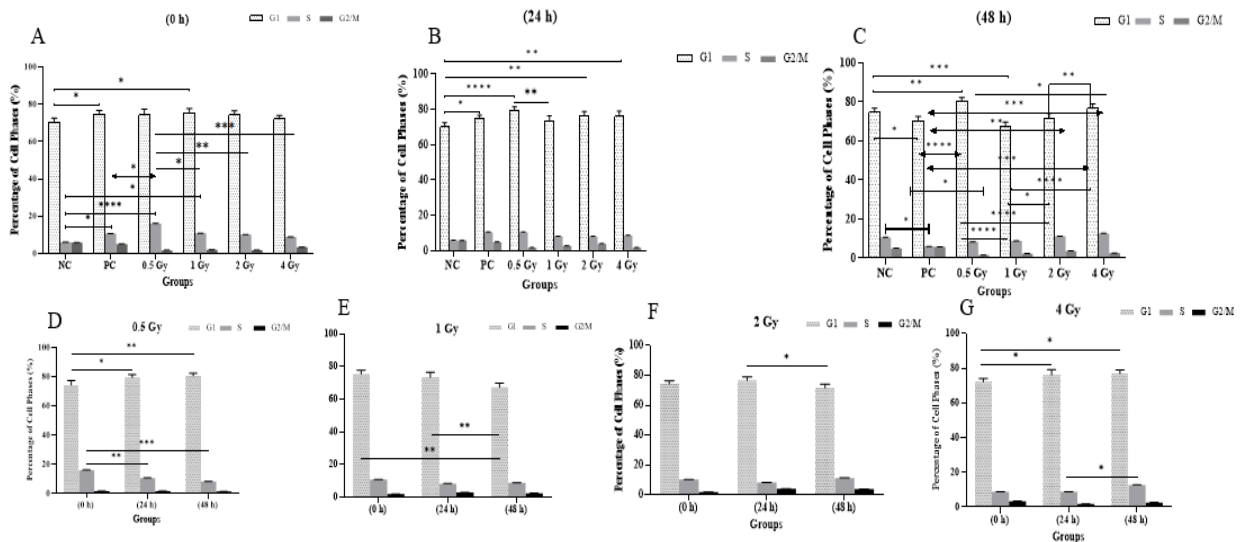


Figure 3. Cell Cycle Phase Distribution in MDA-MB-231 Cells: effect of radiation-conditioned medium (RCM). (A–C) Percentage of cells in G1, S, and G2/M phases across different RCM dose groups at 0 h (A), 24 h (B), and 48 h (C). (D–G) Time-dependent changes within individual RCM dose groups: 0.5 Gy (D), 1 Gy (E), 2 Gy (F), and 4 Gy (G). Cell cycle distribution was analyzed by flow cytometry following PI staining. Data are presented as mean $\pm$ SD from three independent experiments. Statistical analysis was performed using two-way ANOVA followed by Tukey's post hoc test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

in the NC and PC groups ($P < 0.05$). In the same groups, these increases were constant and even became higher at 24 and 48 hours, suggesting an ongoing upregulation over time. By contrast, the 4 Gy RCM group showed no appreciable change at any one-time point, indicating that high-dose radiation could either repress or fail to trigger *miR-31*-associated bystander signaling (Figure 5).

Discussion

Radiation-induced bystander effects (RIBEs) represent a paradigm shift in understanding the biological responses to ionizing radiation, extending beyond direct DNA damage in irradiated cells to include alterations in neighboring non-irradiated cells. The present study aimed to elucidate the impact of radiation-conditioned media (RCM) derived from irradiated MCF-7 breast cancer cells on non-irradiated MDA-MB-231 cells, with a particular focus on the modulation of cell viability, apoptosis, migration, cell cycle dynamics, and *miR-31* expression [18, 21, 22]. Our findings offer valuable insights into the intricate mechanisms underlying bystander responses and highlight a potential role for microRNAs, particularly *miR-31*, in regulating these effects.

MTT assay results demonstrated a modest but statistically significant reduction in the viability of MDA-MB-231 cells exposed to RCM collected from 2 Gy and 4 Gy irradiated MCF-7 cells, particularly at the 0-hour and 24-hour time points. These reductions, although relatively small (2–4%), were consistently observed and reached statistical significance compared to both negative and positive control groups. This finding is consistent with prior studies indicating that bystander factors released from irradiated cells can impair the metabolic activity of neighboring cells. However, the modest magnitude of viability loss suggests that RCM-associated bystander

effects may primarily function through sublethal stress mechanisms rather than overt cytotoxicity, especially at low to moderate radiation doses.

Flow cytometry analysis revealed a precise dose- and time-dependent increase in apoptosis in RCM-exposed MDA-MB-231 cells. Exposure to RCM from 2 Gy and 4 Gy irradiated cells significantly elevated apoptotic rates at both 24 and 48 h, whereas minimal apoptotic induction was observed in the 0.5 Gy group. These findings align with previous reports demonstrating that radiation-induced intercellular signaling can promote apoptosis in bystander cells. Notably, the temporal increase in apoptosis paralleled the sustained upregulation of *miR-31*, suggesting a potential association between *miR-31* upregulation and apoptotic responses in this context. This is supported by Körner et al. [23], who reported that *miR-31* sensitizes breast cancer cells to apoptosis by directly inhibiting PKC ϵ , a known anti-apoptotic regulator. The clear dose-dependency observed in our study further emphasizes the notion that a threshold level of radiation-induced stress is necessary to initiate robust bystander apoptotic responses. While low-dose exposures (0.5 Gy) may elicit subclinical or reversible alterations, higher doses appear to trigger irreversible cell death programs in non-irradiated cells.

The migratory capacity of MDA-MB-231 cells was significantly enhanced following exposure to RCM from irradiated cells at 0.5 Gy and 2 Gy, particularly at 24 hours' post-treatment. However, this promotive effect diminished by 48 h, and no significant migration enhancement was detected in the 4 Gy group. These findings suggest that low-to-moderate doses of RCM promote the transient activation of migration pathways, potentially through soluble signaling molecules, extracellular vesicle-associated factors, or microRNAs present in radiation-conditioned medium. Interestingly, the observed

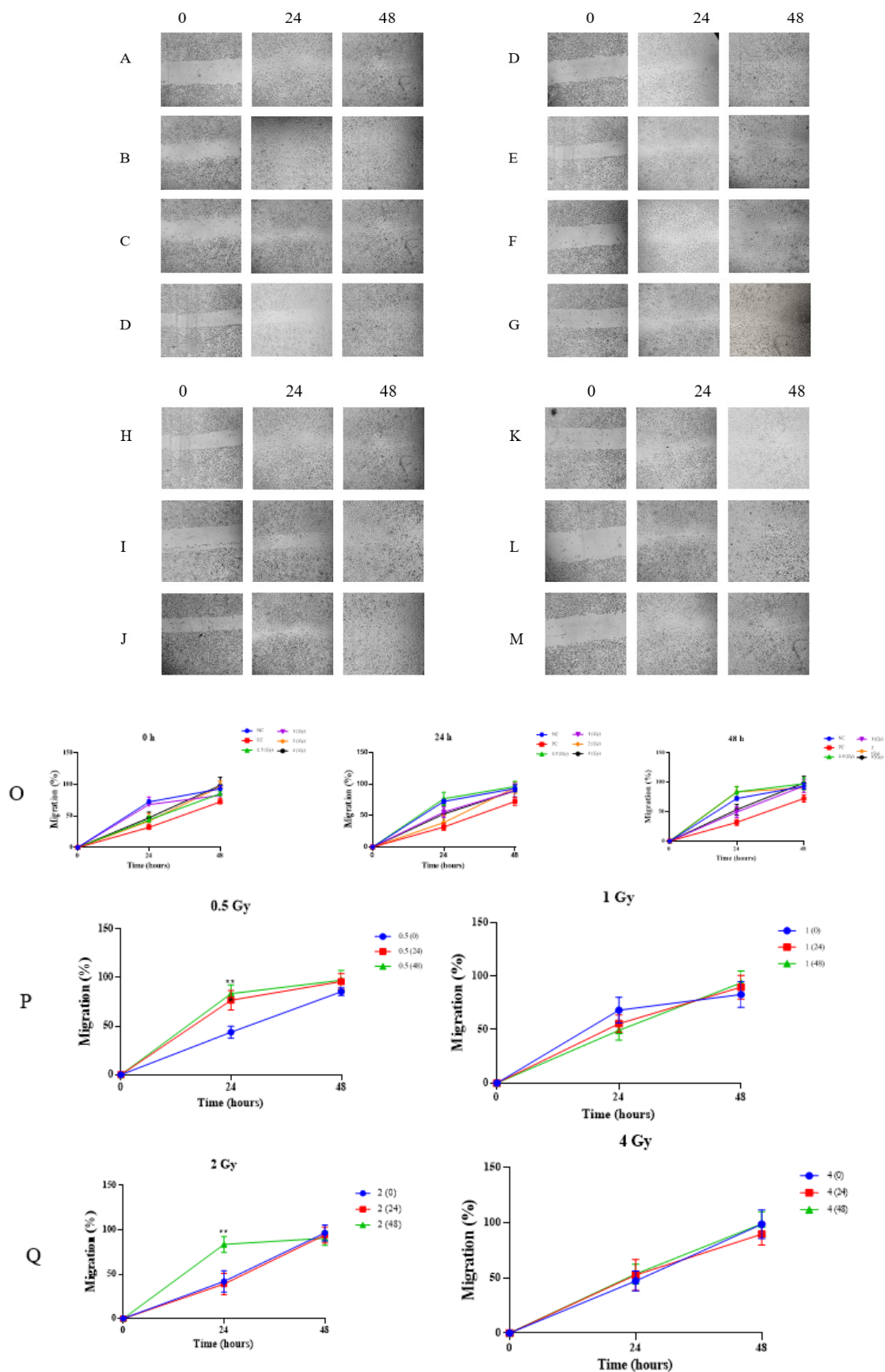


Figure 4. Effect of RCM on Migration Percentage of MDA-MB-231 Cells throughout Time and Wound Closure. (A–M) Representative phase-contrast images of wound healing assays in MDA-MB-231 cells exposed to RCM collected from irradiated MCF-7 cells at 0, 24, and 48 h. (O) Comparison of migration percentages among all RCM groups at different time points. (P–S) Time-dependent migration changes within individual RCM dose groups: 0.5 Gy (P), 1 Gy (Q), 2 Gy (R), and 4 Gy (S). Migration was quantified using ImageJ software. Data are presented as mean $\pm$ SD from three independent experiments. Statistical analysis was performed using two-way ANOVA followed by Tukey's post hoc test. *p < 0.05, **p < 0.01.

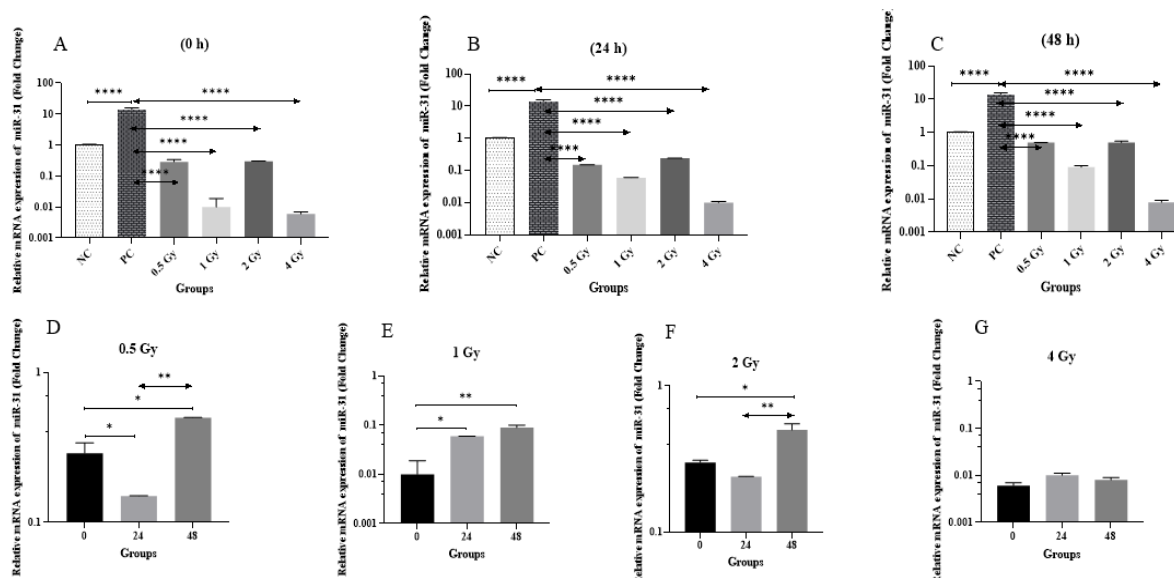


Figure 5. Relative expression of *miR-31* in MDA-MB-231 cells exposed to RCM. (A–C) Fold changes in *miR-31* expression across different RCM dose groups at 0 h (A), 24 h (B), and 48 h (C). (D–G) Time-dependent variations in *miR-31* expression within individual RCM dose groups: 0.5 Gy (D), 1 Gy (E), 2 Gy (F), and 4 Gy (G). Expression levels were normalized to Snord-48. Data are presented as mean $\pm$ SD from three independent experiments. Statistical analysis was performed using two-way ANOVA followed by Tukey's post hoc test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

enhancement in migration correlated with upregulated *miR-31* expression, which has been previously shown to modulate cytoskeletal organization and motility [24, 25]. However, in breast cancer models, elevated *miR-31* levels have been more commonly associated with inhibition of metastasis and migration suppression. This apparent discrepancy may reflect the context-dependent nature of *miR-31* signaling and the complex composition of radiation-conditioned medium, which contains multiple soluble and vesicle-associated signaling molecules. Therefore, the observed migratory responses are unlikely to be exclusively attributable to *miR-31* expression alone.

MDA-MB-231 cells. At 0 h, S-phase accumulation was predominantly observed in cells exposed to 0.5 Gy RCM, suggesting a transient replicative stress response. In contrast, prolonged exposure (24 and 48 h) to higher-dose RCM (2 Gy and 4 Gy) resulted in G1 phase arrest and a corresponding reduction in S-phase entry. These findings are consistent with prior studies, which report that *miR-31* can inhibit cell cycle progression by targeting key regulators, such as HDAC2 and CDK2. The dose-dependent shift from S-phase accumulation to G1 arrest suggests that low-dose RCM may initially disrupt DNA synthesis checkpoints. At the same time, higher doses

promote a more robust cell cycle blockade at the G1/S transition [26–28].

Perhaps the most striking aspect of our study was the consistent and significant upregulation of *miR-31* expression in MDA-MB-231 cells following exposure to 0.5, 1, and 2 Gy RCM, but not in cells exposed to 4 Gy RCM. This dose-specific regulation suggests that *miR-31* upregulation appeared to be more pronounced at moderate radiation doses. In contrast, higher doses may be associated with reduced *miR-31*-related signaling responses, oxidative stress overload or suppression of miRNA biogenesis pathways. *miR-31* has been extensively studied in the context of cancer biology, with dual roles reported: it functions as both a tumor suppressor and an oncogene, depending on the cellular context. In breast cancer, elevated *miR-31* has generally been associated with reduced metastatic potential and increased radiosensitivity, which aligns with our observations of increased apoptosis and altered migration following RCM exposure [13, 29, 30].

Moreover, the parallel patterns between *miR-31* upregulation and phenotypic changes observed in our study suggest a possible association between *miR-31* expression and radiation-induced bystander responses.

Table 1. Primers Sequences.

Gene name	Forward seq (5'-3')
Universal Reverse	GTGCAGGGTCCGAGGT
mi-31 loop	GTTGGCTCTGGTGCAGGGTCCGAGGTATTCGCACCAGAG
mi-31 primer	GTTTAGGCAAGATGCTGGC
UniRprimer	GTGCAGGGTCCGAGGT
Snord 48	GTTGGCTCTGGTGCAGGGTCCGAGGTATTCGCACCAGAGCCAACGGTCAG
Snord 48-F	GTAGTGATGATGACCCAG

However, definitive mechanistic validation through knockdown or overexpression experiments remains necessary to confirm causality.

While our study provides valuable insights into the bystander responses elicited by RCM, several limitations should be acknowledged. First, we did not perform functional assays to directly modulate *miR-31* levels. In addition, exosomes were not isolated or characterized in the present study; therefore, the contribution of exosome-associated signaling could not be directly confirmed. Furthermore, no *miR-31* knockdown, overexpression, or rescue experiments were performed to validate the functional role of *miR-31* in the observed phenotypic changes. Also, we primarily focused on short-term outcomes (up to 48 h) and did not assess potential long-term consequences of RCM exposure, such as genomic instability, senescence, or secondary transformation. Finally, although we utilized breast cancer cell lines with distinct molecular profiles (MCF-7 and MDA-MB-231), further studies using normal tissue cells and in vivo models are required to clarify the clinical relevance of radiation-induced bystander effects.

Future studies should focus on dissecting the molecular pathways downstream of *miR-31*, validating key target genes, and exploring the therapeutic use of miRNA mimics or inhibitors to modulate bystander effects. Additionally, investigating the differential impact of radiation quality (e.g., photons, protons, and heavy ions) on bystander signaling could provide novel insights into optimizing radiotherapy protocols.

In conclusion, in this study, we show that non-irradiated MDA-MB-231 breast cancer cells show notable bystander effects from radiation-conditioned medium (RCM) derived from irradiated MCF-7 cells. In a dose- and time-dependent manner, exposure to RCM caused modest but quantifiable decreases in cell viability, encouraged apoptosis, changed cell cycle distribution, and modified migratory capability. Furthermore, we showed that low-to-moderate-dose RCM regularly elevated *miR-31* expression, suggesting a potential association between altered *miR-31* expression and the observed bystander-related cellular responses. Further mechanistic and in vivo studies are required to clarify the precise biological significance of *miR-31*-associated signaling in radiation-induced bystander effects. Mechanistic research and in vivo validation, among other further investigations, are justified to clarify the exact molecular pathways involved and investigate the therapeutic possibilities of changing bystander signaling in cancer therapy.

Author Contribution Statement

The study's conception and design were the result of contributions from all authors. SSMA, RGHB, AD, MR, and NCH carried out material preparation, data collection, and analysis. RGHB and AD wrote the initial draft of the manuscript. All authors provided feedback on earlier versions of the manuscript, and all authors reviewed and approved the final manuscript.

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Declarations

The Human Ethics Committee of Ahvaz Jundishapur University of Medical Sciences (IR.AJUMS.MEDICINE.REC.1400.033) approved the experimental protocols. They were conducted according to the ethics guidelines of the national health and research institutes.

Availability of data and materials

The datasets utilized and/or examined in the present investigation can be obtained from the corresponding author upon a reasonable request.

Competing interests

The authors declare that they have no conflicts of interest.

References

- Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin.* 2018;68(6):394-424. <https://doi.org/10.3322/caac.21492>.
- Ghanbarnasab Behbahani R, Danyaei A, Teimoori A, Tahmasbi MJ, Neisi N. CRISPR/Cas9 mediated knocking out of OPN gene enhances radiosensitivity in MDA-MB-231 breast cancer cell line. *J Cancer Res Clin Oncol.* 2023;149(7):4117-30. <https://doi.org/10.1007/s00432-022-04304-7>.
- Tagliafico AS, Piana M, Schenone D, Lai R, Massone AM, Houssami N. Overview of radiomics in breast cancer diagnosis and prognostication. *Breast.* 2020;49:74-80. <https://doi.org/10.1016/j.breast.2019.10.018>.
- Xu S, Wang J, Ding N, Hu W, Zhang X, Wang B, et al. Exosome-mediated microRNA transfer plays a role in radiation-induced bystander effect. *RNA Biol.* 2015;12(12):1355-63. <https://doi.org/10.1080/15476286.2015.1100795>.
- Reynders K, Illidge T, Siva S, Chang JY, De Ruyscher D. The abscopal effect of local radiotherapy: Using immunotherapy to make a rare event clinically relevant. *Cancer Treat Rev.* 2015;41(6):503-10. <https://doi.org/10.1016/j.ctrv.2015.03.011>.
- Le M, Fernandez-Palomo C, McNeill FE, Seymour CB, Rainbow AJ, Mothersill CE. Exosomes are released by bystander cells exposed to radiation-induced biophoton signals: Reconciling the mechanisms mediating the bystander effect. *PLoS One.* 2017;12(3):e0173685. <https://doi.org/10.1371/journal.pone.0173685>.
- Zhang D, Zhou T, He F, Rong Y, Lee SH, Wu S, et al. Reactive oxygen species formation and bystander effects in gradient irradiation on human breast cancer cells. *Oncotarget.* 2016;7(27):41622-36. <https://doi.org/10.18632/oncotarget.9517>.
- Moghassemi S, Dadashzadeh A, Sousa MJ, Vlieghe H, Yang J, Leon-Felix CM, et al. Extracellular vesicles in nanomedicine and regenerative medicine: A review over the last decade. *Bioact Mater.* 2024;36:126-56. <https://doi.org/10.1016/j.bioactmat.2024.02.021>.

9. Thery C, Witwer KW, Aikawa E, Alcaraz MJ, Anderson JD, Andriantsitohaina R, et al. Minimal information for studies of extracellular vesicles 2018 (MISEV2018): A position statement of the International Society for Extracellular Vesicles and update of the MISEV2014 guidelines. *J Extracell Vesicles*. 2018;7(1):1535750. <https://doi.org/10.1080/20013078.2018.1535750>.
10. Thakur BK, Zhang H, Becker A, Matei I, Huang Y, Costa-Silva B, et al. Double-stranded DNA in exosomes: A novel biomarker in cancer detection. *Cell Res*. 2014;24(6):766-9. <https://doi.org/10.1038/cr.2014.44>.
11. Li S, Shao L, Xu T, Jiang X, Yang G, Dong L. An indispensable tool: Exosomes play a role in therapy for radiation damage. *Biomed Pharmacother*. 2021;137:111401. <https://doi.org/10.1016/j.biopha.2021.111401>.
12. Tang S, Cheng J, Yao Y, Lou C, Wang L, Huang X, et al. Combination of four serum exosomal MiRNAs as novel diagnostic biomarkers for early-stage gastric cancer. *Front Genet*. 2020;11:237. <https://doi.org/10.3389/fgene.2020.00237>.
13. Yu T, Ma P, Wu D, Shu Y, Gao W. Functions and mechanisms of microRNA-31 in human cancers. *Biomed Pharmacother*. 2018;108:1162-9. <https://doi.org/10.1016/j.biopha.2018.09.132>.
14. Zhang W, Zhu Y, Zhou Y, Wang J, Jiang P, Xue L. miRNA-31 increases radiosensitivity through targeting STK40 in colorectal cancer cells. *Asia Pac J Clin Oncol*. 2022;18(3):267-78. <https://doi.org/10.1111/ajco.13602>.
15. Gao Y, Yi J, Zhang K, Bai F, Feng B, Wang R, et al. Downregulation of *miR-31* stimulates expression of LATS2 via the hippo pathway and promotes epithelial-mesenchymal transition in esophageal squamous cell carcinoma. *J Exp Clin Cancer Res*. 2017;36(1):161. <https://doi.org/10.1186/s13046-017-0622-1>.
16. Smolarz M, Skoczylas L, Gawin M, Krzyzowska M, Pietrowska M, Widlak P. Radiation-induced bystander effect mediated by exosomes involves the replication stress in recipient cells. *Int J Mol Sci*. 2022;23(8):4169. <https://doi.org/10.3390/ijms23084169>.
17. Jassi C, Kuo WW, Kuo CH, Chang CM, Chen MC, Shih TC, et al. Mediation of radiation-induced bystander effect and epigenetic modification: The role of exosomes in cancer radioresistance. *Heliyon*. 2024;10(14):e34460. <https://doi.org/10.1016/j.heliyon.2024.e34460>.
18. McAndrews KM, Kalluri R. Mechanisms associated with biogenesis of exosomes in cancer. *Mol Cancer*. 2019;18(1):52. <https://doi.org/10.1186/s12943-019-0963-9>.
19. Jafari A, Babajani A, Abdollahpour-Alitappeh M, Ahmadi N, Rezaei-Tavirani M. Exosomes and cancer: From molecular mechanisms to clinical applications. *Med Oncol*. 2021;38(4):45. <https://doi.org/10.1007/s12032-021-01491-0>.
20. Davoren PA, McNeill RE, Lowery AJ, Kerin MJ, Miller N. Identification of suitable endogenous control genes for microRNA gene expression analysis in human breast cancer. *BMC Mol Biol*. 2008;9(1):76. <https://doi.org/10.1186/1471-2199-9-76>.
21. Hinzman CP, Baulch JE, Mehta KY, Girgis M, Bansal S, Gill K, et al. Plasma-derived extracellular vesicles yield predictive markers of cranial irradiation exposure in mice. *Sci Rep*. 2019;9(1):9460. <https://doi.org/10.1038/s41598-019-45970-x>.
22. Jeyaram A, Lamichhane TN, Wang S, Zou L, Dahal E, Kronstadt SM, et al. Enhanced loading of functional miRNA cargo via PH gradient modification of extracellular vesicles. *Mol Ther*. 2020;28(3):975-85. <https://doi.org/10.1016/j.ymthe.2019.12.007>.
23. Körner C, Keklikoglou I, Bender C, Wörner A, Münstermann E, Wiemann S. MicroRNA-31 sensitizes human breast cells to apoptosis by direct targeting of protein kinase C ϵ (PKC ϵ). *J Biol Chem*. 2013;288(12):8750-61.
24. Maggisano V, Capriglione F, Verrienti A, Celano M, Sponziello M, Pecce V, et al. Expression of *miR-31*-5p affects growth, migration and invasiveness of papillary thyroid cancer cells. *Endocrine*. 2023;79(3):517-26. <https://doi.org/10.1007/s12020-022-03267-6>.
25. Lin X, Wu W, Ying Y, Luo J, Xu X, Zheng L, et al. MicroRNA-31: A pivotal oncogenic factor in oral squamous cell carcinoma. *Cell Death Discov*. 2022;8(1):140. <https://doi.org/10.1038/s41420-022-00948-z>.
26. Citrin DE. Recent developments in radiotherapy. *N Engl J Med*. 2017;377(11):1065-75. <https://doi.org/10.1056/NEJMr1608986>.
27. Baskar R, Lee KA, Yeo R, Yeoh KW. Cancer and radiation therapy: Current advances and future directions. *Int J Med Sci*. 2012;9(3):193-9. <https://doi.org/10.7150/ijms.3635>.
28. Bhatnagar N, Li X, Padi SKR, Zhang Q, Tang MS, Guo B. Downregulation of miR-205 and *miR-31* confers resistance to chemotherapy-induced apoptosis in prostate cancer cells. *Cell Death Dis*. 2010;1(12):e105. <https://doi.org/10.1038/cddis.2010.85>.
29. Yang SJ, Yang SY, Wang DD, Chen X, Shen HY, Zhang XH, et al. The miR-30 family: Versatile players in breast cancer. *Tumour Biol*. 2017;39(3):1010428317692204. <https://doi.org/10.1177/1010428317692204>.
30. Augoff K, McCue B, Plow EF, Sossey-Alaoui K. *miR-31* and its host gene lncRNA LOC554202 are regulated by promoter hypermethylation in triple-negative breast cancer. *Mol Cancer*. 2012;11(1):5. <https://doi.org/10.1186/1476-4598-11-5>.



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