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REVIEW

Emerging role of microRNAs in breast cancer radiotherapy

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Breast cancer represents the leading cause of death in women worldwide. Ionizing radiation is one of the most relevant therapeutic approaches for the treatment of this type of cancer. Unfortunately, either resistance of tumor cells to therapeutic doses of radiation or normal tissue tolerance have proven to limit the effectiveness of radiotherapy. In the last few years, several studies have highlighted an important link between radioresistance, cancer and microRNAs (miRNAs), an emerging class of endogenous non coding RNAs that control gene expression. MiRNAs influence carcinogenesis at multiple stages and effectively control tumor radiosensitivity as they affect levels of target genes regulating relavant radio-related signal transduction pathways. Since radiation- and multidrug-resistances are the characteristic properties of numerous type of tumors, there is a huge interest in understanding the connection between miRNA expression in tumors and chemo- or radio-sensitivity. In the present review, we summarize the emerging evidence of miRNAs involvement in the radioresponse of breast tumors and discuss their potential role of radiosensitizers.

Keywords: radiation therapy; breast cancer; miRNA

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Introduction

Breast Cancer (BC) constitutes a heterogeneous tumor type entity characterized by a complex spectrum of malignant features and clinical outcomes. The optimal management of BC requires a multidisciplinary approach and Radiation Therapy (RT) still represents one of the most effective treatment modalities. Unfortunately, efficacy of radiation treatment is limited by intrinsic radioresistance of cancer cells, which correlates with an increase in the risk of tumor local recurrence [1].

How to reduce tumor radioresistance and improve radiosensitivity is indeed a hot topic in the field of RT, which explains the high interest in identifying new molecules endowed with synergistic effects with radiation.

Recently, the discovery of microRNAs (miRNAs), a class of functional, non-coding small RNAs has changed the landscape of cancer biology [2]. MiRNAs (generally 21 to 24 nucleotides in length) are a large family of non-coding RNAs acting as post-transcriptional regulators of gene expression. MiRNA genes are transcribed by RNA polymerase II as large primary transcripts (pri-miRNA) that are processed by a protein complex containing the RNase III enzyme Drosha, to form an approximately 70 nucleotide precursor microRNA (pre-miRNA). Such precursor is then transported to the cytoplasm where it is processed by the RNase III enzyme DICER, to form a mature miRNA of approximately 22 nucleotides. The mature miRNA is then incorporated into a ribonuclear particle to form the RNA-induced silencing complex (RISC), which binds the 3' untranslated region (3' UTR) of the mRNA and mediates gene silencing. For more detailed mechanistic knowledge on miRNA mechanism of

action, we indeed recommend to the readers other specialized reviews [2, 3, 4].

The most deleterious damage induced by ionizing radiation (IR) is thought to be the double strand breaks (DSBs) of DNA, resulting from the generation of excision repair breaks opposite each other on the two DNA strands, and by the formation of an excision repair break opposite a DNA daughter-strand gap [5]. If unrepaired, DSBs may affect cell survival, as they lead to chromosome aberrations, genomic instability and even cell death [6]. Human cells respond to DNA damage by activating the DNA damage response (DDR), a molecular machinery which detects and repairs DNA damage. DSBs are processed either by non-homologous end joining (NHEJ) or homologous recombination (HR): while NHEJ promotes the potentially inaccurate ligation of DSBs, HR precisely restores the genomic sequence of the broken DNA ends by exploiting sister chromatids as template for repair. DNA repair is carried out by a plethora of enzymatic activities aimed at repairing the DNA damage, which include several enzymes such as nucleases, helicases, polymerases, recombinases, ligases and many others [7,8].

MiRNAs regulate almost every cellular pathway, including the DDR [9]. A high interest in the applications of miRNAs in radiation oncology is due to their deep involvement in the DDR process [10].

How specific miRNAs regulate cancer radioresistance and can be used as radiosensitizing agents is now a matter of investigation. Therefore, understanding the correlation between miRNAs and RT will provide possible improvements in the current standard therapy of BC.

MiRNAs influence RT by affecting specific cellular pathways

The cellular exposure to IR triggers various physiological responses including DNA damage processing, cell cycle arrest, differentiation, signal transduction alterations, mutations, gene expression modifications, genomic instability and induction of carcinogenesis. Increased evidence supports a role for miRNAs in regulating key cellular pathways involved in response to radiation [11].

Recent studies have demonstrated that miRNAs influence many stages of carcinogenesis and can effectively modulate tumor radiosensitivity at different levels, by affecting targets involved in DNA damage repair, cell cycle checkpoint, apoptosis, signal transduction pathways and tumor microenvironment [12, 13].

The most common injury produced by IR is DSB, then cancer cells activate several signaling pathways to repair DNA damage. Noteworthy, tumor cells may utilize both the two major pathway, i.e. the NHEJ or the HR, to repair DNA breaks.

MiRNAs are involved in regulating at the post-transcriptional level the expression of important targets in the DDR pathway [14, 15]. Indeed, miRNAs have been shown to efficiently modulate tumor radiosensitivity by blocking both the NHEJ and HR repair pathways involved in the DDR; they have proven to interfere with the major pathways activated by ionizing radiation, such as the PI3-K/Akt, NF-κB, MAPK and TGFβ signaling pathways [16].

Herein, many examples of well-characterized miRNAs involved in these processes are discussed.

Lal *et al*. showed that miRNAs down-regulate DSB repair factors and suppress DNA repair in terminally differentiated blood cells [17]. BRCA1 plays essential roles in HR, NHEJ and nucleotide excision repair, by interacting with components of the DNA repair machinery and regulating expression of genes involved in DDR pathways [18]. However, BCRA1 deficiency also determines sensitivity to DNA-damaging agents such as PARP inhibitors. The over-expression of miR-182, through the use of miRNA mimics or by a lentiviral vector, induced a down-regulation of BCRA1 with the consequent enhancement of *in vitro* radiosensitivity of BC cells. On the basis of these findings, the authors postulated that miR-182-dependent targeting of BRCA1 hampers DNA repair in BC cells [19].

The chromatin remodeling complex SNF2H (also known as SMARCA5) has been shown to regulate both the HR and NHEJ DNA repair pathways. In a study by Mueller *et al*., it has been shown that the down-regulation of miR-99 family members might represent a mechanism by which cancer cells acquire resistance to DNA damage by increasing DNA repair efficiency and consequently making cancer cells more resistant to RT. IR-induced miR-99 up-regulation reduced the capability of cells to repair DNA damage by regulating the expression of SNF2H and increasing radiosensitivity of MCF7 BC cells [20].

RAD51 and RAD52 represent other two essential proteins implicated in DSBs repair and HR. RAD51 plays the critical role of catalyzing the transfer of the strand, between a broken sequence and its undamaged homolog, to resynthetize the damaged DNA region. MiR-155 was shown to target the 3′-UTR of RAD51, and miR-155 over-expression *in vitro* and *in vivo* regulated DNA repair activity and sensitivity to IR by

repressing RAD51 in BC, inducing a delay in repair after IR exposure [21]. Another miRNA, miR-302a, inversely correlated with AKT1 and RAD52 expression levels; inhibition of miR-302 conferred radioresistance while miR-302 over-expression sensitized BC cells to RT [22].

The Ser/Thr kinase ataxia telangiectasia mutated (ATM) is the principal signaling molecule in response to DSBs. The activation of ATM occurs within minutes of a DSB, independently of the cell cycle's phase [23]. ATM is the main player in DDR initiating the cascade leading to cell cycle checkpoint activation and DNA repair [24]. Importantly, ATM is directly regulated by several miRNAs [25,26], whose over-expression was found to reduce ATM expression and to radio-sensitize different tumor cell lines, including BC cell lines.

Song *et al*. showed that ATM protein kinase was targeted by miR-18a; furthermore, they demonstrated that over-expression of miR-18a down-regulated ATM expression by targeting the ATM-3'-UTR, thus resulting in a reduction of both DDR and HRR efficiencies, and in an increase in cellular radiosensitivity to IR treatment [27]. A recent study by Zhang *et al*. has indicated that miR-205 promoted radiosensitivity and that it was down-regulated in radioresistant subpopulations of BC cells; loss of miR-205 highly associated with poor distant relapse-free survival in BC patients. MiR-205 inhibited DDR and radiosensitized tumor cells by targeting ZEB1 and Ubc13; in turn, radiation suppressed miR-205 expression through ATM and zinc finger E-box binding homeobox 1 (ZEB1), a crucial epithelial to mesenchymal transition (EMT) activator by inhibiting E-cadherin expression [28, 29]. EMT is essential in cancer invasion, metastatic dissemination and drug-resistance [30]. Moreover, miR-205 targeted the human epidermal growth factor receptor 3 (HER3), a receptor tyrosine kinase of the epidermal growth factor receptor (EGFR) family, thereby inactivating the downstream signals mediated by Akt, suppressing the phosphoinositide 3-kinase (PI3K)/Akt pathway and inhibiting BC proliferation with better responses to targeted therapies [31, 32].

The role of miR-200c in the EMT process has been extensively studied. Up-regulation of miR-200c inhibited cancer aggressiveness, metastases and chemoresistance in some tumors [33, 34]. Lin *et al*. found out that BC cells bearing high expression of miR-200c displayed higher radiosensitivity. MiR-200 was also shown to directly target TANK-binding kinase (TBK1), which directly activates AKT [35]. Given the well known association between the PI3-K/AKT pathway and the radiation resistance mechanisms [36], a direct inhibition of such pathway led to an increased radiosensitivity of cancer cells.

Specific histone modifications are essential steps of DDR [9]. To allow DNA repair, the repair proteins have to be recruited and to gain access to the site of DNA damage, which is normally packed inside a nucleosome. The histone variant H2AX is phosphorylated by ATM (γH2AX) in response to DSB and is part of the cascade that leads to DNA repair [37]. Presumably, γ-H2AX foci attract repair factors, leading to their accumulation at DSB site. Specific recognition of γ-H2AX by these repair factors requires the presence of protein domains, which bind to the phosphorylated carboxy-terminus of γ-H2AX. MiR-138 targets H2AX [38]; over-expression of miR-138 led to higher chromosomal breaks and sensitivity to IR and cytotoxic drugs in the BC cell line MDA-MB231. H2AX with miR let-7 and caspases are also targets of Lin28, a marker of cancer stem cells, which is implicated in the mechanism of radioresistance of lung, pancreatic and BC cells. Wang *et al*. provided evidence that Lin28 expression was up-regulated in radiation-resistant BC cells and that Lin28 transfection induced radiation resistance; furthermore, the authors showed that over-expression of Lin28 and radiation effectively induced H2AX expression and inhibited γ-H2AX and p21 expression, while let-7 overexpression enhanced sensitivity to radiation [39].

Cell cycle checkpoints are generally activated by DNA damage. One of the key proteins in the checkpoint pathways is represented by the tumor suppressor gene p53 [40], which coordinates DNA repair with cell cycle progression and apoptosis. P53 mediates the two major DNA damage-dependent cellular checkpoints, one at the G_1/S transition and the other at the G_2/M transition. G_2/M, together with the latter part of the S phase, is the most radiosensitive phase of the cell cycle. For this reason, RT may be more effective with a partial synchronization in such radiosensitive phases of the cell cycle [41]. Anastasov *et al*. studied the regulation of cellular response to RT by miR-21-mediated modulation of cell cycle progression in BC cells. MiR-21 is considered a promising target in radiation therapy [42], especially for the possible functional link between miR-21 and the p53 tumour suppressor pathway, where p53-induced proteins evoke apoptosis in response to DNA damage after irradiation. In this study, miR-21 was reported to promote radioresistance of BC cells by inducing G_2/M cell cycle arrest; anti-apoptotic effects triggered by miR-21 expression was also evident after IR exposure and correlated with radioresistance. In addition, miR-21 influenced cell cycle progression via DNA damage-G_2 checkpoint induction; in this way, miR-21 inhibition by antagomiRs was able to reduce the G_2/M block and to potentiate apoptotic cell death induction after RT [43].

One of the direct targets of p53 is miR-34, and it has been shown that miR-34 plays a role in cell cycle arrest, inhibition of proliferation and apoptosis. After radiation injury, several miRNA profiling strategies detected miR-34 family members as upregulated in different types of human cell lines [9, 44], as well as in the nematode *Caenorhabditis elegans* model system [45].

miRNAs as predictive biomarkers in BC-RT

Since RT remains one of the most important methods for the treatment of BC, there is a great interest in identifying factors which might help to predict patients' response to treatment. Importantly, predicting the response to RT, by distinguishing between radioresistant and radiosensitive patients, could be helpful to minimize the risk of unnecessary treatment and the consequently related side effects. Recent studies have demonstrated the potential role of miRNAs as predicting biomarkers of patient response to different treatments including chemotherapy and RT [46, 47]. The potential of miRNAs to correlate with response to a given treatment, and their remarkable stability in blood and serum, make miRNAs novel potential diagnostic and prognostic biomarkers in the personalized management of BC [48].

Accumulating evidence is underlying the possible contribution of miRNAs as valuable biomarkers to predict response to chemotherapy [49]. For instance, down-regulation of miR-34, miR-17 and miR-7a associated with chemosensitivity to fluorouracil, adriamycin and cyclophosphamide, respectively [50]. In preclinical studies employing BC cell lines, miR-21 inhibition correlated with increased sensitivity to topotecan and taxol [51]. Moreover, circulating miR-210 and miR-125b were associated with sensitivity to trastuzumab and resistance to neo-adjuvant chemotherapy, respectively [52, 53].

On the other hand, few clinical studies have demonstrated the impact of miRNAs to RT response. In a recent study by Halimi *et al*., the hypothesis of a close direct or inverse correlation between miRNAs and different proteins expressed in BC cells was tested. Several proteins, such as vascular endothelial growth factor (VEGF), EGFR, human epidermal growth factor receptor-2 (Her-2/neu), B-cell lymphoma 2 (BCL-2) and p53, are involved in breast tumour cell radiosensitivity and radioresistance. Given the well acknowledged concept that these proteins do not have the same expression pattern in radiosensitive and radioresistant BC patients, different expression of their related miRNAs was actually expected. Starting with the analysis of levels of circulating miRNAs in blood samples of patients before and after RT, the authors demonstrated that miR-21 and miR-206 were up-regulated while miR-7, -34a, -29, -15 and -16 down-regulated in radioresistant BC patients, suggesting that it may be possible to use circulating miRNAs to predict patient response to RT [54] .

In another study, Sochor *et al*. evaluated the expression of miR-155, miR-19a, miR-181b, miR-24, relative to let-7a in the sera of 63 patients with early BC (EBC) and 21 healthy controls. These miRNAs were selected because they target key molecules involved in tumor growth and aggressiveness of BC. Analysis was conducted collecting patient serum in diverse phases of the disease: diagnosis, after surgery, and after treatment with chemotherapy and radiotherapy. The data indicated that high-risk EBC patients (classified using current clinical prognostic factors including HER2, Ki-67 and grade III) possessed higher serum levels of oncomiRs than female-healthy controls, while low-risk patients tended to have low expression of oncomiRs as compared to the high-risk ones. Finally, levels of these four BC pathogenesis-related oncomiRs dramatically decreased after combined treatment, suggesting their potential for the EBC patient monitoring [55].

The above-mentioned miR-205, known as a down-regulated miRNA in radioresistant BC cells, is also correlated with tumor recurrence and distant relapse (metastasis) in breast cancer. Zhang *et al*. performed a retrospective analysis on a cohort of human BC patients in which miRNA profiling was obtained from 207 tumor samples; 84% of these patients received RT. It was observed that patients with low miR-205 expression levels (defined as the bottom 20%) in their tumors had much worse distant relapse-free survival than those with high miR-205 [28]. The same trend was observed for miR-21, which seems to be a molecular prognostic marker for BC progression. Moreover, Yan *et al*. showed that miR-21 over-expression correlated with specific BC bio-pathologic features, such as advanced tumor stage, lymph node metastasis and poor survival of patients [56]. In another study, Halimi *et al*. investigated serum levels of miR-21 in BC patients after RT (25 sessions of radiotherapy with a total of 50 Gy irradiation) as well as in 20 healthy volunteers. MiR-21 levels before RT were comparable within healthy volunteers but increased significantly after RT of BC patients, discriminating irradiated and not-irradiated patients with high specificity (75%) and sensitivity (80%), thus demonstrating its role as a biomarker of IR exposure [57] .

Among BC subtypes, triple negative BC (TNBC) is characterized by a poorer survival compared to the others. Gasparini *et al*. first highlighted the correlation between miR-155 and the prognosis of TNBC patients: in a sub-cohort of 93 TNBC patients treated with RT alone or

with chemotherapy plus RT, miR-155 levels positively correlated with the overall survival of patients [21].

Conclusions

In recent years, many reports have demonstrated an important role of miRNAs in determining the response of cancer cells to radiations. On the light of the published data, miRNAs modulating DDR and/or signaling pathways tightly connected to cancer cell radioresistance could represent particularly useful targets in the contexts of novel experimental strategies aimed at modulating radioresponsivity [16].

Results obtained in BC cell lines have demonstrated a direct involvement of selected miRNAs in determining the radiation response, thus revealing a new mechanism by which tumors may be refractory to RT. MiRNAs, such as miR-21 [43], miR-99 [20], miR-155 [21], miR-138 [38] and many others, each regulating key players in different radiation-relevant pathways, could represent particularly useful weapons for the development of strategies aimed at modulating radioresponsivity.

However, there is a strong need to confirm results obtained in BC cell lines in adequate preclinical models which recapitulate the disease scenario in order to draw a significant conclusion on the role of miRNAs in the onset of BC cell radioresistance as well as on their clinical utility as novel cancer radiosensitizers.

Another intriguing feature of circulating miRNAs is their potential as novel biomarkers of radiation response: notably, they can predict the radioresistance of tumors before treatment, monitor the response through the treatment, and also define RT endpoints along with risks of BC recurrence [54,58]. However, data have been so far obtained in very small and heterogeneous groups of BC patients treated with different RT-based regimens, which obviously limits the current usefulness of miRNAs as predictive markers for a more precise stratification of patients responding to RT.

In conclusion, research performed thus far supports a relevant role for miRNAs in the future of radiation oncology, which could hopefully provide the basis for the development of miRNA-based customized treatments to enhance radiosensitivity or to predict response of BC patients to RT.

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Conflicts- of- interest disclosure

The authors declare no competing financial interests.

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