Correction

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The editors wish to note that, after this article was published, a reader noticed that some fragments of text and some sentences in the abstract, significance statement, introduction, and discussion overlap with text from other articles and were reproduced without quotation marks. No concerns have been raised about the originality of the research or about the results and conclusions.

Some text in the abstract and the significance statement overlaps with text from ref. 19.


Some text in the introduction overlaps with text from the following references:


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Protective role of miR-155 in breast cancer through RAD51 targeting impairs homologous recombination after irradiation

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Contributed by Carlo M. Croce, February 12, 2014 (sent for review December 9, 2013)

Cell survival after DNA damage relies on DNA repair, the abrogation of which causes genomic instability and development of cancer. However, defective DNA repair in cancer cells can be exploited for cancer therapy using DNA-damaging agents. DNA double-strand breaks are the major lethal lesions induced by ionizing radiation (IR) and can be efficiently repaired by DNA homologous recombination, a system that requires numerous factors including the recombinase RAD51 (RAD51). Therapies combined with adjunctive radiotherapy have been demonstrated to improve the survival of triple-negative breast cancer patients; however, such therapy is challenged by the emergence of resistance in tumor cells. It is, therefore, essential to develop novel therapeutic strategies to overcome radioresistance and improve radiosensitivity. In this study we show that overexpression of microRNA 155 (miR-155) in human breast cancer cells reduces the levels of RAD51 and affects the cellular response to IR. miR-155 directly targets the 3′-untranslated region of RAD51. Overexpression of miR-155 decreased the efficiency of homologous recombination repair and enhanced sensitivity to IR in vitro and in vivo. High miR-155 levels were associated with lower RAD51 expression and with better overall survival of patients in a large series of triple-negative breast cancers. Taken together, our findings indicate that miR-155 regulates DNA repair activity and sensitivity to IR by repressing RAD51 in breast cancer. Testing for expression levels of miR-155 may be useful in the identification of breast cancer patients who will benefit from an IR-based therapeutic approach.

DNA repair plays a critical role in preventing the development of cancer, whereas defective DNA repair in cancer cells can be exploited for cancer therapy using DNA-damaging agents (1–3). Trustworthy genome transmission requires a complex network of pathways, including: DNA replication, recognition and signaling of DNA damage, cell-cycle checkpoint activation, DNA repair/recombination, and programmed cell death. In response to DNA damage, cells arrest their cell-cycle progression, thus providing time for repair, or depending on extent of damage, activate programmed cell death, both responses preventing transmission of genetic instability (4, 5). DNA double-strand breaks (DSBs) are the major lethal lesions induced by ionizing radiation (IR) and can be efficiently repaired by the error-free process of DNA homologous recombination (HR) or by nonhomologous end-joining recombination, a potentially error-prone process (6–8).

HR is the critical pathway for the error-free repair of DNA DSBs, mainly occurring in S and G2 phases of the cell cycle (9). HR requires several synergistically operating factors, including the RAD51 recombinase (RAD51) and the breast/ovarian cancer susceptibility gene products: breast cancer 1, early onset (BRCA1) and breast cancer 2, early onset (BRCA2) (10, 11).

RAD51 is a central protein in homologous recombination, playing the critical role of catalyzing the transfer of the strand, between a broken sequence and its undamaged homolog to resynthesize the damaged region (6, 7). Dysregulation of RAD51 can lead to impaired HR and aberrant genome rearrangements, genoevents that are often observed in cancers (12). Germ-line mutations in RAD51 would probably be too damaging to be tolerated, as supported by the observation that disruption of the RAD51 gene leads to embryonic death (13).

RAD51 activity is affected by interactions with many other proteins that form the HR molecular machine (7): BRCA2 allows the translocation of RAD51 into the nucleus (14, 15), tumor protein p53 (p53) inhibits or reverts the RAD51-dependent DNA strand exchange process (15, 16), and Bcr/Abl or Bcl-2 overexpression leads to inhibition of the RAD51 pathway (17, 18). Many other proteins can affect RAD51 activity by acting upstream of this cascade or by posttranscriptional regulation of RAD51 expression levels (19).

Conflicting reports have been published on the role of RAD51 dysregulation in breast carcinogenesis. Some studies have reported concomitant down-regulation of BRCA1 and increase of RAD51 expression in breast cancer tissue (20, 21); however, others have reported that expression of RAD51 is decreased in breast cancer (22). The authors declare no conflict of interest.

Data deposition: The miRNA microarray expression data have been deposited in the Gene Expression Omnibus (GEO) database, www.ncbi.nlm.nih.gov/geo (accession no. GSE41970).

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levels in sporadic invasive ductal breast cancer, and others reported reduced levels of both proteins in breast tumor cell lines and breast cancer cells, leading to divergent speculation about the role of RAD51 in this type of tumor (12, 20–23).

MicroRNAs (miRNAs) are small (19–25 nt) noncoding RNAs that reduce the abundance and translational efficiency of mRNAs and play a major role in regulatory networks, influencing diverse biological processes through effects of individual miRNAs on translation of multiple mRNAs (24, 25).

Our group has previously demonstrated the pro-oncogenic role of microRNA 155 (miR-155) in leukemogenesis and the role of miR-155 in the mismatch repair DNA repair pathway through the targeting of MSH2, MSH6, and MLH1 in colon cancer (26, 27). We have recently focused our studies on triple-negative breast cancer (TNBC), for which we generated a prognostic miRNA signature, including miR-155, in a large cohort of TNBCs (28). So far only a few reports about miRNA’s role in breast cancer (29) agreed with our finding of a protective effect of miR-155 in breast cancer. We further investigated this protective role of miR-155 because it is potentially very relevant for the outcome of breast cancer patients. miR-155 acts as protector through its role in the DNA damage process.

In this study, we identify RAD51 as a target of miR-155 and characterize the protective role of miR-155 in the HR process in TNBC.

Results

miR-155 Is a Prognostic Factor in the TNBC Model. TNBCs account for 15–20% of newly diagnosed breast cancer cases and are clinically defined by the lack of expression of estrogen receptor, progesterone receptor, and the absence of amplification or overexpression of HER2 (30). In general, patients with TNBC present larger, higher-grade tumors, increased numbers of involved nodes, and poorer survival compared with other cancer subtypes. TNBC treatment has been challenging owing to the absence of well-defined molecular targets.

We previously showed the correlation between expression of an miRNA signature and the prognosis of TNBC (28). Among the signature miRNAs, miR-155 expression could significantly stratify TNBC patients according to prognosis. Based on data availability we considered miR-155 expression in a subcohort of 93 TNBCs treated with both chemotherapy plus radiation and with radiation only (see Table S1 for demographic characteristics of the subcohort and ref. 31 for the characteristics of the whole cohort); miR-155 expression level positively correlated with the overall survival for patients ($P = 0.031$, hazard ratio $2.24$, 95% confidence interval (CI) 1.08–4.51; Fig. L4). This finding was further confirmed in an independent non-TNBC breast cancer cohort by interrogating the MIRUMiR database (29) (Fig. S1).

Because IR is mainly used for its ability to induce DSBs, we investigated the effect of miR-155 overexpression on HR downstream of BRCA1, which is a major player in breast cancer susceptibility but is also the initiator protein of the HR cascade (7) action.

RAD51 Is a Direct Target of miR-155. We used in silico prediction models to identify potential binding sites for miR-155 in the mRNA of the genes involved in HR. Two putative sites were found in RAD51 using RNAhybrid (BiBiServ; NCBI NM_002875.4): one in the coding sequence (CDS) (Fig. 1B, Upper) and the other in the 3′-UTR (Fig. 1B, Lower).

As a functional screen, we subcloned the coding sequence together with the 3′-UTR region (including the predicted miR-155 seed regions) of RAD51 in the multicloning site of the psiche2 vector (Promega) using the primers reported in Table S2 and recorded luciferase activity (32). 293HEK cells were chosen for their low basal level of miR-155 and high transfection efficiency and were transfected with the luciferase reporter constructs and miR-155 precursor (premiR-155) or control precursor (premiR control) at 100 nM. We observed luciferase activity reduction of 47.0% in the presence of premiR-155 plus the construct containing the entire CDS–3′-UTR region, 41.4% reduction activity with the same construct lacking the miR-155 CDS binding site, and only 4.1% reduced activity with the mutant of the 3′-UTR binding site (Fig. 1C). The deletion of the 3′-UTR binding site for miR-155 restored the luciferase activity almost completely, indicating that this is the site through which the miR-155 exerts its action against RAD51 ($P < 0.001$).

To test this finding in vitro, we infected MCF7 breast cancer cell lines with a lentiviral miR-155 overexpression vector (miR-155) and with miRZip-155 construct from SBI (System Biosciences) to knock down the miR-155 expression (anti-miR-155). As expected, ectopic miR-155 expression induced significant RAD51 down-regulation (Fig. S2, Upper Left); the result was further confirmed by real-time PCR (Fig. S2, Lower Left). The transfection with the miRZip-155 to knock down the miRNA expression was not apparently very efficient, but the Western blot (Fig. S2, Upper Right) shows the up-regulation of RAD51 after miR-155 silencing (densitometry was performed with ImagequantTN); both these results were confirmed by real-time PCR as shown in Fig. S2, Lower Right. We further assessed the miR-155/RAD51 anticorrelation in 14 different breast cancer-derived cell lines; Fig. 2A shows the expected anticorrelation between miR-155 and RAD51 expression levels.

Mir-155 Correlates with Better Prognosis Through RAD51 Targeting in TNBC. RAD51 expression in breast cancer was investigated through the Oncomine database and gene microarray data analysis tool. A

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**Fig. 1.** miR-155 protective role in TNBC through RAD51 targeting. (A) Overall survival of TNBC patients according to miR-155 expression; the cohort was dichotomized using the median expression as cutoff. (B) Positions of miR-155 putative binding sites on RAD51 transcript. (C) Psiche2 vector with RAD51 WT insert (full-length RAD51 transcript) with Mut mir-155 CDS and Mut mir-155 3′-UTR containing a deletion of the miR-155 target site in the CDS (position 367) and in the 3′-UTR (position 1594), respectively, were cotransfected with mir-155 or scrambled mir in 293HEK cells. Luciferase activity was recorded after 24 h. Data represent the mean ± SD from at least three determinations from four independent transfections.
meta-analysis of gene expression in the breast cancer TCGA (The Cancer Genome Atlas) microarray studies (33) pinpointed RAD51 as significantly up-regulated in tumor samples (Fig. S3A, P < 0.001) and in TNBC tumors in comparison with conventional-type breast cancers (Fig. S3B, P < 0.01).

RAD51 immunohistochemistry scores together with miRNA expression levels were assessed in a cohort of 134 TNBCs (Fig. 2). miR-155 expression levels were directly and significantly anti-correlated with the RAD51 immunohistochemical H-score (Fig. 2B, P = 0.008). Furthermore, in lymph node metastatic samples a significant RAD51 down-regulation correlated with a significant miR-155 up-regulation was observed in comparison with the primary matched tumors (Fig. 2C, P = 0.001; Fig. 2D, P < 0.001; and Fig. 2E).

The 134 patients were stratified equally into four groups based on the expression levels of miR-155 and RAD51 immunohistochemical H-score (each group contains 25% of the patients) for further statistical analysis.

High expression of miR-155 together with low expression of RAD51 was significantly associated with increased overall survival of patients in 77 samples (P = 0.043, hazard ratio 0.48, 95% CI 0.24–0.98; Fig. 2F).

**miR-155 Inhibits HR and Enhances Cellular Sensitivity to IR.** Definitive local therapies combined with adjuvant radiotherapy have been demonstrated to improve the survival of TNBC patients (34, 35). However, such therapy is challenged by the emergence of resistance in tumor cells. It is, therefore, of paramount importance to develop novel therapeutic strategies to overcome radioresistance and improve radiosensitivity. Enhanced DNA repair capacity such as HR repair leads to resistance to radiotherapy (36); we therefore investigated in vitro the effects of the miR-155/RAD51 interaction in the HR pathway.

The miR-155 influence on cell reproductive death after treatment with IR, as assessed by clonogenic assay, was determined using stably transfected MCF7 cells (MCF7 scrambled and MCF7 miR-155) after different IR doses (2, 4, 6, and 8 Gy). Although not striking, there was a significant reduction in the survival fraction of colonies following IR in the MCF7 miR-155–transfected cells compared with the scramble-transfected ones (Fig. S4A, P = 0.046).

To further assess the effects of the exogenous overexpression of miR-155 on RAD51 function, we performed the HR assay. HeLa-DR13-9 cells [a gift from J. D. Parvin (Ohio State University, Columbus, OH) and M. Jasin (Memorial Sloan Kettering Cancer Center, New York)] were used as a “vessel” to determine whether miR-155 can inhibit or enhance the HR process following the protocol of Ransburgh et al. (37).

HeLa-DR13-9 cells were transfected with scrambled and miR-155 oligo from Ambion. siRNAs against RAD57 and BRCA1 genes (ON-TARGETplus SMARTpool; Thermofisher) were also used as positive controls. miR-155 overexpression dramatically reduced the homologous recombination process by 62%; similar effects were obtained after the forced silencing of key repair genes, including RAD51 itself (71.6%) and BRCA1 (92.1%) (Fig. 3B).

**miR-155 Is a Negative Regulator of RAD51 Foci Formation.** We evaluated the effects of miR-155 ectopic overexpression on RAD51 foci formation after IR. We performed this experiment in MCF10A and MCF7 cell lines; only MCF7 data are shown for convenience, but MCF10A results were comparable.

MCF7 stably expressing miR-155 (MCF7 miR-155) or control precursor (MCF7 Scrambled) were gamma-irradiated (8 Gy) and then stained for immunofluorescence (IF) studies or used for protein and RNA collection.

A consistent increase in miR-155 expression level compared with the scrambled miRNA control was determined by quantitative PCR for all of the stably overexpressing cell lines (Fig. S2). Fig. 44 shows a representative IF image of RAD51 foci in MCF7 cells at 30 min after IR; the miR-155 overexpression is shown by GFP-positive cells, and RAD51 foci are visibly diminished in highly GFP-positive cells. Counting of RAD51 foci formation at different time points after 8-Gy IR shows a statistically significant decrease in RAD51 foci formation in cells stably transfected with miR-155 compared with control precursor (Fig. 4B, P < 0.001), which showed a significant up-regulation of RAD51 foci at 3 h after IR (Fig. 4B).

**miR-155 Targeting of RAD51 Induces a Delay in DNA Repair.** We further evaluated the effects of miR-155 ectopic overexpression as positive controls. miR-155 overexpression dramatically reduced the homologous recombination process by 62%; similar effects were obtained after the forced silencing of key repair genes, including RAD51 itself (71.6%) and BRCA1 (92.1%) (Fig. 3B).

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on phosphorylated histone family member X (γ-H2AX), which forms nuclear foci at sites of DNA damage, facilitating DNA damage response and repair following IR (38, 39).

γ-H2AX foci detected by IF were significantly higher in miR-155 transfected cells before irradiation and a significant down-regulation was observed after 30 min and 1 hr from IR exposure (Fig. 5A, P < 0.001). Counts of γ-H2AX foci at later time points show a significant increase in miR-155-overexpressing cells, suggesting a delay in DNA damage repair induced by the miR-155 targeting of RAD51 (Fig. 5A and B).

The results were further confirmed by flow cytometry using the AlexaFlour488 γ-H2AX (Ser139) conjugated antibody in transfected cell lines 24 h after IR exposure (8 Gy) (Fig. 5C, P < 0.001). Cells transfected with miR scrambled, after 8-Gy irradiation, had a much lower percentage of active foci compared with cells that overexpressed miR-155, further supporting the IF data.

RAD51 and γ-H2AX levels were investigated at three different time points after IR exposure by Western blot (Fig. 6). Significant RAD51 down-regulation owing to the miR-155 overexpression is observed at 4 h after IR exposure, when RAD51 is induced by IR exposure owing to activation of the repair complex, whereas only small differences are observed at 30 min and 2 h. This probably means that RAD51 is an essential protein (also confirmed by the fact that knockdown of RAD51 causes embryonic death) and that the targeting of miR-155 is taking place significantly only over “basal expression levels” (e.g., only after its induction by IR). To demonstrate this observation we transfected cells with Origene expression vector pCMV6-XL4-RAD51 full length together with oligo miR-155 and oligo miR scrambled. In this condition RAD51 down-regulation was evident already 30 min after IR. Furthermore, the transfection of pCMV6-XL4-RAD51-mut (3′-UTR miR155 binding site deleted) did not show as strong RAD51 targeting by miR-155, further confirmation of the 3′-UTR binding site’s essential role. miR-155 transfected cell lines showed persistently high levels of γ-H2AX signal at 4 h after IR compared with the scrambled, indicating a delay in the repair process, still ongoing in miR-155 overexpressing cells.

Discussion

TNBC is a heterogeneous disease; there are no specifically targeted therapies for the treatment of this particularly aggressive type of cancer, resulting in poor outcome for patients. Radiation therapy is often administered postsurgery without any real data on its benefits. Finding molecular markers that can predict the benefits of radiation therapy for TNBC patients can save discomfort and toxicity to patients, as well as reduce treatment costs. We previously reported the pro-oncogenic properties of miR-155 in leukemogenesis and colon cancer, but the opposite behavior was found after studying the TNBC model (28), where miR-155 overexpression was defined as protective.

This intriguing feature of miR-155 pushed us to further investigate the reasons for its prosurvival behavior in the TNBC model.

DNA DSBs are typically repaired by BRCA1/2-initiated-mediated HR, which is mostly deficient in triple-negative tumors.

The DNA damage response process is frequently impaired in aggressive breast cancers, as a consequence of either mutation or deregulation of critical components, such as BRCA1, ataxia telangiectasia mutated (ATM), and p53. Whereas p53

Fig. 4. miR-155 inhibits gamma-rays-induced RAD51 foci formation. (A) Stably miR-155–overexpressing MCF7 cells were treated with IR (8 Gy) and then fixed for immunofluorescent staining of RAD51, 30 min after IR. Representative image of RAD51 immunostaining (Upper Left); GFP signal represents the miR-155 overexpression in the stable clones (Upper Right); Lower Left represents the counterstain with DAPI; Lower Right shows the overlay of the three signals. (B) Bar graph shows relative quantification of RAD51 foci in cells with at least 10 foci after IR (8 Gy) at different time points; miR-155 overexpression induces a decrease in RAD51 foci formation compared with scrambled clones.

Fig. 5. miR-155 increases gamma-rays-induced γ-H2AX foci formation. (A) Bar graph shows relative quantification of γ-H2AX foci in cells with at least 10 foci after IR (8 Gy) at different time points; overall miR-155 overexpression induced a marked increase in γ-H2AX foci compared with scrambled clones. (B) Stably miR-155–overexpressing MCF7 cells were treated with IR (8 Gy) and then fixed for immunofluorescent staining of γ-H2AX foci, 30 min after IR. Representative image of γ-H2AX immunostaining in scrambled stably transfected MCF7 cells is shown Upper Right; Upper Left shows an increase in γ-H2AX foci in MCF7 stably overexpressing miR-155. Lower panels represent the respective counterstain with DAPI. (C) Graph shows the detection of γ-H2AX by flow cytometry (phospho-histone H2AX, Alexa Fluor 488 conjugate). MCF7 24 h after transfection with scrambled or miR-155 were exposed to IR (8 Gy) and harvesting at different time points. Data are normalized to untransfected and unirradiated cells.
mRNA expression. Gasparini et al. www.oncomine.org (46), was explored (August 1, 2013) for RAD51 mRNA expression in the TCGA breast cancer series. Oncomine algorithms were used for the statistical analysis of the differences in RAD51 mRNA expression.

Patients. An institutional review board-approved protocol [Cancer Institutional Review Board of The Ohio State University (OSU)] for this research linked clinical features, treatment, and outcome data of breast cancer patients in the OSU National Comprehensive Cancer Network breast cancer database/tumor registry with archival breast cancer pathology specimens stored in the OSU Tissue Archive Service using the Information Warehouse at OSU Medical Center to serve as “honest broker” and provided deidentified clinical/pathological information. No consent was required because the clinical data stored in the OSU Tumor Registry and pathologic specimens stored in Path Archives were deidentified. Consecutive triple-negative localized breast cancer patients (n = 365) were identified from 1995 to 2005. After pathology review for tumors with sufficient sample for study, 173 paraffin blocks for TNBCs were identified for preparation of a tissue microarray and cores for RNA preparation. For preparation of RNA, we used two 1.75-mm cores for tumor and for normal and two 0.6-mm cores (28) were taken for preparation of the tissue microarray in duplicate. Institutional review board approval was obtained for the use of these human tissues and associated anonymous data.

miRNA Expression and Statistical Analysis. Total RNAs were processed with the nanoString Counter system (nanoString) in the Nucleic Acid Shared Resource of OSU. The miRNA panel detects 664 endogenous miRNAs (with 654 probes), 82 putative viral miRNAs, and 5 housekeeping transcripts. See ref. 28 for expanded methods and validation of the microarray results.

The miRNA microarray expression data have been deposited in the Gene Expression Omnibus (GEO) database with accession number GSE41970. All fold changes associated with these analyses are represented in log2 scale (logFC).

Statistical analysis results are expressed as mean ± SD unless indicated otherwise. Comparisons between groups were performed using the two-tailed Student t test or Benjamini-Hochberg method. Significance was accepted at a P value <0.05. Graphpad Prism version 5.0 was used for Pearson correlations.

More detailed information is provided in SI Methods.

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