Oncogenic miR-181a/b affect the DNA damage response in aggressive breast cancer

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Breast cancer is a heterogeneous tumor type characterized by a complex spectrum of molecular aberrations, resulting in a diverse array of malignant features and clinical outcomes. Deciphering the molecular mechanisms that fuel breast cancer development and act as determinants of aggressiveness is a primary need to improve patient management. Among other alterations, aberrant expression of microRNAs has been found in breast cancer and other human tumors, where they act as either oncogenes or tumor suppressors by virtue of their ability to finely modulate gene expression at the post-transcriptional level. In this study, we describe a new role for miR-181a/b as negative regulators of the DNA damage response in breast cancer, impacting on the expression and activity of the stress-sensor kinase ataxia telangiectasia mutated (ATM). We report that miR-181a and miR-181b were overexpressed in more aggressive breast cancers, and their expression correlates inversely with ATM levels. Moreover we demonstrate that deregulated expression of miR-181a/b determines the sensitivity of triple-negative breast cancer cells to the poly-ADP-ribose-polymerase1 (PARP1) inhibition. These evidences suggest that monitoring the expression of miR-181a/b could be helpful in tailoring more effective treatments based on inhibition of PARP1 in breast and other tumor types.

Introduction

The relevance of the DNA damage response (DDR) pathway in providing a cell-intrinsic barrier against cancer progression has clearly emerged in the last years. Experimental and clinical data indicated that DDR activation occurs at early stages of transformation as a consequence of oncogene deregulation, and bypassing its growth-suppressive outcomes (apoptosis or senescence) is required for cancer progression. Consequently, cancer cells are under positive selective pressure for DDR inactivation, as frequently observed in breast cancer, where inherited inactivating mutations of critical DDR components including ATM and the breast cancer susceptibility gene 1 and 2 (BRCA1/2) predispose to the development of hereditary breast carcinomas. In contrast, in sporadic breast cancers, which account for nearly 90% of all mammary tumors, ATM and BRCA1 mutations are detected in only 2% of cases (www.sanger.ac.uk/genetics/CGP/cosmic). Nonetheless, reduced expression and activity of BRCA1 and ATM are frequent events in sporadic breast tumors. This has been reported to occur as a consequence of either promoter methylation, deregulated transcriptional control or aberrant regulation by microRNAs (miRNAs). In particular, down-regulation of ATM and/or BRCA1 has been frequently observed in more aggressive breast cancers, such as the Basal-like and triple-negative (TNBC, i.e., ER−/PR−/HER2− tumors) breast cancers subtypes. These two groups of tumors show a high degree of overlap and frequently display a phenotype defined “BRCAness” that is characterized by traits similar to BRCA-mutated breast tumors, including lack of estrogen receptor, high grade, aggressiveness and frequent TP53 mutations. Despite this role in malignancies, the molecular basis of BRCAness is still largely unclear.

Filling this gap in knowledge would be of particular relevance from a therapeutic perspective, since deficiency in proteins involved in the DDR and in DNA double-strand break repair by homologous recombination (HR) is considered a major determinant of response to chemotherapy. For instance, ATM or BRCA1-deficient tumors display an extreme sensitivity to radiotherapy and chemotherapeutic agents (i.e., platinum-derivates), and a selective “synthetic lethal” effect can be achieved with the pharmacological inhibition of the DNA repair protein poly (ADP-Ribose) polymerase 1 (PARP1).

In addition to genetic and epigenetic changes, aberrant post-transcriptional modulation of gene expression by miRNAs is emerging among major factors contributing to the unbalance of oncogenes and tumor suppressors in human cancers. miRNAs

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are small RNAs that finely regulate gene expression at the post-transcriptional level by interacting with the 3’UTR of their target transcripts through partial sequence complementarity, strongly contributing to tumorigenic hallmarks of breast cancer, including stemness, deregulated proliferation, genomic instability and metastatic potential, and recently it has been suggested that miRNAs directly targeting BRCAness (e.g., miR-182 and miR-146) might be involved in establishing BRCAness traits.

In this study, we highlight a role for miR-181a/b in determining the BRCAness phenotype in aggressive breast cancers. We demonstrate that miR-181a/b negatively impact on ATM levels and activity and dampen DDR, thereby conferring to breast cancer cells highly expressing miR-181a/b a sensitivity to treatment with the PARP1 inhibitor Olaparib.

Results

Increased expression of miR-181a/b correlates with breast cancer aggressiveness. To identify miRNAs that may be deregulated in breast cancer, we performed a survey of public breast cancer data sets for miRNA expression. This search highlighted that miR-181b is frequently overexpressed in tumor samples included in three different breast cancer collections (Fig. S1). To validate this data, we investigated the expression of miR-181b and of its sibling miR-181a (originated from the same polycistrionic transcripts) in a panel of 104 snap-frozen primary breast cancers and eight normal tissue counterparts (104 BC data set, Table S1). As shown in Figure 1A, higher levels of miR-181a/b were detected in tumor samples as compared with normal tissue, and, importantly, their expression levels correlated positively with tumor aggressiveness, with grade 3 (G3) tumors showing highest expression of the two miRNAs with respect to grade 1 (G1) and 2 (G2) tumors (Fig. 1A) as well as with proliferation index, as judged by KI67 protein expression (Fig. 1B). In particular, in G3 tumors, high levels of miR-181a/b were detected in 67% (miR-181a) and 70% (miR-181b) of cases, while in G1 samples, only 29% of them showed high levels of expression for miR-181a and 18% of them for miR-181b (Fig. 1C).

We next investigated the association of miR-181 expression (stratified by tertiles) with the clinical outcome in a cohort of 123 primary breast cancers with annotated clinical history (123 BC, Table S2). Of note, high expression levels of miR-181b were associated with shorter disease-free survival (Fig. 1D), with a nearly 3-fold increased risk of developing recurrence (HR = 2.89, Cox univariate regression analysis, 95% CI 1.36, 6.16, p < 0.006). Accordingly, primary tumors that gave rise to early metastatic events within 5 y since diagnosis (“relapse”) displayed more frequent overexpression of miR-181b (“high” expression) as compared with non-metastatic primary tumors (“disease-free,” Fig.1E).

Altogether, these data obtained in the clinical setting suggest that miR-181a/b play a role in promoting breast cancer aggressiveness.
MCF10A cells transformed with RASV12 upon stable overexpression of miR-181a/b (Fig. S6A and B). Following DNA damage, ATM promotes the phosphorylation and activation of several factors, among them the BRCA1 tumor suppressor. Phosphorylation of BRCA1 on Serine 1524 after DNA damage was reduced upon overexpression of miR-181a/b (Fig. 3E), while it was increased upon inhibition of endogenous miR-181a/b (Fig. 3F).

The above results suggest that aberrant overexpression of miR-181a/b may have a major impact on the cellular response to DNA damage. In particular, we evaluated the impact of miR-181a/b overexpression on the efficiency of DNA repair by HR by monitoring the appearance of RAD51 foci as a consequence of γ-radiation. Notably, the number of foci-containing cells 4 h after irradiation was lower upon overexpression of miR-181a/b as compared with controls in two different cell lines, and this effect is similar to that observed upon knocking down ATM expression by RNAi (p < 0.05, Fig. 4A and C; Fig. S7, black columns). Conversely, 48 h after DNA damage, cells overexpressing miR-181a/b were still highly positive for RAD51 foci, while controls showed only a residual signal, thus suggesting that the unscheduled expression of miR-181a/b leads to persistence of unrepaired DNA lesions (p < 0.001, Fig. 4B and C; Fig. S7, gray columns).

In summary, the above results indicate that miR-181a/b are able to dampen both DDR and the DNA repair process by HR in breast cancer cells, causing the accumulation of unfixed lesions in DNA.

**miR-181a/b act as determinants of sensitivity to PARP inhibition.** Defects in proteins involved in DNA double-strand breaks repair and HR (e.g., ATM and BRCA1/2) display synthetic lethality with inhibition of base excision repair factors, such as poly (ADP-ribose) polymerase (PARP) 1. Based on our evidence, we reasoned that overexpression of miR-181a/b could sensitize breast
effects were observed in a panel of different cancer cell lines of breast (SUM159PT), ovarian (OVCAR3), pancreas (PANC1) and colon (HT29) origin (Fig. 5C; Figs. S10 and 11).

Finally, we sought to demonstrate whether endogenous miR-181a/b expression could mediate the sensitivity of these cells to PARP inhibition. To this aim, SUM159PT cells were transfected with antagomiRs against miR-181a and miR-181b and then treated with Olaparib. As reported in Figure 5D, inhibition of endogenous miR-181a/b significantly decreased cell death caused by Olaparib treatment as compared with control, and this effect was concomitant with the rescue of ATM expression. Altogether, these results indicate that deregulated expression of miR-181a/b sensitizes cancer cells to PARP inhibition.

Discussion

Breast cancer is a heterogeneous disease that comprises a range of distinct tumor types differing in their biological and clinical.
Our study now demonstrates that deregulation of miR-181a/b expression, by dampening the DDR, represents a key mechanism to establish BRCAness traits in aggressive breast cancer. High levels of miR-181a/b may dampen ATM and BRCA1 functions in tumors bearing functional alleles of these genes. We demonstrated that ATM levels are strongly downregulated by miR-181a/b in breast cancer cells, in agreement with previous findings by Wang et al., who identified the miR-181 family as TGF-β-regulated miRNAs that contribute to the expansion of breast cancer stem-like cells by acting on ATM levels. We provide evidence that miR-181a/b are overexpressed in breast cancer, where they correlate with aggressive features, that they are associated with the likelihood to develop distant metastases (as also recently reported by Taylor and colleagues) and, most notably, we found an inverse correlation between miR-181a/b and ATM expression in human breast tumors. Accordingly, we found that miR-181a/b overexpression impairs the proper induction of both features. The most aggressive breast cancer subtypes, such as the Basal-like and triple-negative breast cancer, are characterized by high rates of relapse and of chemoresistant metastasis and greatly suffer from lack of therapeutic options. Several evidences indicate that the DNA damage response process is frequently impaired in more aggressive breast cancers, as a consequence of either mutation or altered expression of critical components, such as BRCA1, ATM and p53. While p53 mutations are reported with high frequency, mutations in genes coding for ATM or BRCA1 represent rare events in sporadic breast cancers. Nevertheless, functional impairment of BRCA (“BRCAness” phenotype) has been frequently observed in sporadic breast cancers. Likely, the existence of alternative mechanisms curbing the expression and functions of either BRCA1 or its regulators, such as ATM, may underlie this phenomenon. Among these mechanisms, aberrant activity of miRNAs could play a relevant role, as reported for miR-146 and miR-182, which directly target BRCA1.

![Figure 3](image_url)

**Figure 3.** miR-181a/b impairs ATM signaling. (A) MDA-MB-231 cells were transfected with miR-181a, miR-181b, siATM, control siRNA (CTRL) and with a combination of miR-181a and miR-181b inhibitors (IH-miR-181a/b) or control inhibitor (IH-CTRL). After 72 h cells were split, and after 24 h treated with bleomycin (10 μM) for additional 4 h. Cells were then fixed, and immunofluorescence assay was performed to detect cells positive for γH2AX foci. A representative picture is shown for each condition. (B) Graph represents percentage of cells positive for γH2AX foci and shows means and s.d. for three independent experiments. (C) MDA-MB-231 cells transfected with miR-181b or control siRNA (CTRL) and (D) with a combination of miR-181a and miR-181b inhibitors (IH-miR-181a/b) or control inhibitor (IH-CTRL) were treated with bleomycin (10 μM) for 2 or 4 h. Western blot analysis was performed to detect phosphorylated H2AX and vinculin levels (loading control). (E) The same lysates analyzed in (C) were subjected to western blot analysis to detect phosphorylation on serine 1524 of BRCA1, total BRCA1 and vinculin levels (loading control). (F) MDA-MB-231 cells transfected with miR-181a (IH-miR-181a), miR-181b (IH-miR-181b) or control inhibitors (IH-CtRL) were treated with bleomycin (10 μM) for 2 or 4 h and subjected to western blot analysis to detect phosphorylation on serine 1524 of BRCA1, total BRCA1 and vinculin levels (loading control). p values were calculated with two tailed t-test, *, p < 0.05; **, p < 0.01; ***, p < 0.001.
that miR-181a/b expression could also influence the efficacy of platinum-derived compounds. Besides breast cancer, our data suggest that the miR-181a/b-ATM axis may sensitize to the effects of PARP inhibition also in ovarian, pancreatic and colon tumors, where ATM is rarely found mutated (5, 3 and 14%, respectively, according to COSMIC database), while miR-181a/b have been found frequently overexpressed, thus providing an effective mechanism to functionally downregulate ATM, the DDR and the repair of DNA damages also in these tumors.

Therapeutic strategies based on ectopic expression or inhibition of cancer-related miRNAs have been proposed. Although the control of aberrant expression of miR-181a/b and other oncomiR-NAs may represent an appealing therapeutic strategy, several challenges need to be addressed before these approaches could meet clinical application, including tumor delivery, safety and constant target inhibition. An alternative strategy exploiting miRNA deregulation in tumors would be to take advantage of cancer-related miRNAs to predict the clinical response to available treatments. Our data suggest that monitoring miR-181a/b expression alone or in combination with ATM could be a valuable strategy to select patients that would benefit from treatment with PARP inhibitors or platinum-based chemotherapy (both exploiting defects of HR-mediated DNA damage repair), thereby improving the therapeutic options for more aggressive breast cancers.

Figure 4. miR-181a/b alters the assembly of RAD51 foci. (A and B) MDA-MB-231 cells were transfected with a combination of miR-181a/b, ATM (siATM) or control siRNA and treated with 10 Gy of IR. Cells were fixed after 4, 24 and 48 h and immunofluorescence assay was performed to detect cells positive for RAD51 foci. A representative picture is shown for cells fixed after 4 (A) and 48 (B) h. (C) Graph represents percentage of cells positive for RAD51 foci and shows means and s.d. for three independent experiments. *p values were calculated with two-tailed t-test: *, p < 0.05; **, p < 0.01; ***, p < 0.001.

the DDR and the repair of DNA double-strand breaks in breast cancer cells.

miR-181a/b overexpression has been ascribed to aberrant activation of major pathways involved in breast tumorigenesis, including IL6/Stat3, TGF-β, HIF-1, WNT/β-catenin and HMGA1, suggesting that it may represent a crucial mechanism fuelling mammary gland transformation. Remarkably, the miR-181 family has been shown to be deregulated also in several other solid tumors (e.g., pancreas, prostate, gastric and colon) and to be able to target other tumor suppressors, including TIMP3, CYLD, PTEN and p27. Therefore, unscheduled expression of miR-181a/b may represent a common step of cellular transformation, contributing to the acquisition of different hallmarks of cancer.

Our findings have relevant clinical implications for the treatment of basal-like and TNBCs, the most aggressive breast cancer subtype characterized by high rates of relapse, visceral metastases and early death, and for which no effective therapies are available. A subset of TNBCs has shown sensitivity to PARP inhibitors, either alone or in combination with chemotherapy. However, lack of reliable biomarkers other than BRCA1 and BRCA2 to predict sensitivity to PARP inhibitors has curbed the success of phase III clinical trials in TNBC patients. Having shown compelling evidence that the expression of miR-181a/b determines the sensitivity of TNBC cells to the PARP inhibitor Olaparib, we may expect that quantifying the expression of miR-181a/b in basal-like breast cancers or TNBCs could help in predicting the patients’ response to PARP inhibitors. Notably, tumors showing the “BRCAness” traits are also sensitive to platinum-derived anticancer drugs. Since miR-181a expression has been shown to sensitize cancer cells to cisplatin, we may expect that miR-181a/b expression could also influence the efficacy of platinum-derived compounds. Besides breast cancer, our data suggest that the miR-181a/b-ATM axis may sensitize to the effects of PARP inhibition also in ovarian, pancreatic and colon tumors, where ATM is rarely found mutated (5, 3 and 14%, respectively, according to COSMIC database), while miR-181a/b have been found frequently overexpressed, thus providing an effective mechanism to functionally downregulate ATM, the DDR and the repair of DNA damages also in these tumors.

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Materials and Methods

Cell culture, transfections and retroviral transduction. MDA-MB-231, HEK 293GP, MDA-MB-468, SUM159PT, OVCAR, HT29, PANCl and Sk-Br-3 cells were cultured in DMEM medium supplemented with 10% FCS. H1299 cells were
Transfections of H1299 were performed with Lipofectamine 2000 (Invitrogen), following manufacturer’s instructions. For miRNA/siRNA transfections, cells were transfected with 40 nM siRNA oligonucleotides (MWG biotech), 3 nM miRNA (Ambion, PM10421 and PM12442) or 20 nM miRNA inhibitors [Dharmacon IH-300553-07, IH-300553-08, using Lipofectamine RNAiMax (Invitrogen), following manufacturer’s instructions] and cultured in RPMI medium with 10% FCS. MCF10A cells were maintained in DMEM:F12 Ham’s medium 1:1, supplemented with 5% horse serum, insulin (10 μg/ml), hydrocortisone (0.5 μg/ml) and epidermal growth factor (20 ng/ml). All media were added with penicillin and streptomycin antibiotics (100 IU/ml for each). For drug treatments, Bleomycin (Calbiochem 203401) and Olaparib (Selleck AZD2281) were used as indicated.

**Figure 5.** miR-181a/b sensitize cancer cells to Olaparib treatment. (A) Colony formation of MDA-MB-231 cells transfected with miR-181a, miR-181b, ATM (siATM) or control siRNA (CTRL) and treated with 1 μM Olaparib for 10 d. Percentage of surviving fraction are reported. Surviving fractions were calculated as ratio between plating efficiency in Olaparib-treated cells and plating efficiency in untreated cells. Plating efficiency was obtained according the following formula: # of colonies/# of plated cells. (B) MDA-MB-231 cells were transfected with a combination of miR-181a and miR-181b or control siRNA and 72 h hour later splitted and treated with 10 μM Olaparib. After 7 d, cells were harvested and half of them permeabilized and stained with propidium iodide, and the other half stained with PI/Annexin V, and analyzed by flow cytometry. The percentage of sub G population (black columns) and Annexin V-positive cells (gray columns) are reported. (C) SUM159PT, OVCR-3 and PANC1 cells transfected with a combination of miR-181a and miR-181b or control siRNA, were treated with 10 μM Olaparib for 4 (SUM159PT and PANC1) or 7 (OVCR-3) d. Cells were then harvested, stained with propidium iodide and subjected to FACS analysis. The percentage of SubG1 population is reported. A representative western blot showing the levels of ATM and Vinculin as loading control is reported. (D) SUM159PT cells transfected with a combination of antagomiR against miR-181a and miR-181b (ant-181a/b) or a control antagomir mutated in five residues (Ant-181mut), and treated as in (C). A representative western blot showing the levels of ATM and Vinculin as loading control is reported. Graphs show means and s.d. for at least three independent experiments. p values were calculated with two tailed t-test: *, p < 0.05; **, p < 0.01; ***, p < 0.001.
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Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Supplemental Materials
Supplemental materials may be found here: www.landesbioscience.com/journals/cc/article/24757
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