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miR-214 promotes apoptosis and sensitizes breast cancer cells to doxorubicin by targeting the RFWD2-p53 cascade

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Abstract

miR-214 is involved in numerous physiological and pathological processes including tumorigenesis. However, the function of miR-214 in the development and treatment of breast cancer remains elusive. In this study, we report that miR-214 is strikingly down-regulated in breast cancer cell lines and clinical samples, particularly, in the doxorubicin resistant tumor tissues. Remarkably, restoration of miR-214 expression induces apoptosis and sensitizes the MCF7 cells sustaining wild-type p53, but not the p53 null MDA-MB-157 cells, to doxorubicin. Furthermore, we reveal that miR-214 directly down-regulates the expression of RFWD2, also known as COP1, an E3 ligase targeting the tumor suppressor p53 for proteasomal degradation. In addition, RFWD2 protein levels are reversely correlated with miR-214 expression levels in breast cancer tissues. Moreover, ectopic expression of RFWD2 markedly abolishes miR-214-triggered apoptosis of MCF7 cells. In conclusion, miR-214 functions as a tumor suppressor by regulating the RFWD2-p53 cascade, thus delivery of miR-214 analogs could be a potential adjunct therapy in breast cancer harboring wild type p53.
Key words: miR-214, RFWD2, breast cancer, apoptosis, chemotherapy.

1. Introduction

Breast cancer is one of the most common malignant tumors worldwide and the leading cause of cancer death among women [1,2,3]. Chemotherapy is commonly applied to patients resistant to endocrine therapy or advanced stage patients. Doxorubicin is the fundamental and most important drug of the combination chemotherapy regimens [4]. However, breast cancer cells often develop resistance to chemotherapy resulting in a relapse and worsening of prognosis [5]. Therefore, it is of great importance to further unravel the mechanism of induced chemotherapeutic resistance of cancer cells with the ultimate hope of developing novel and effective pharmacological treatments for breast cancer.

Recently, growing evidence has suggested that dysregulation of microRNAs (miRNAs) also contributes to drug resistance [6,7]. miRNAs are a group of non-coding RNA with the length of 18-25 nucleotides involved in many biological processes such as survival, apoptosis, cell cycle and gene regulation [8]. It has been shown that miR-214 is deregulated in multiple human cancers and displays contrasting behavior as oncogenic protein or tumor suppressor [9]. Derfoul A et al. revealed that decreased miR-214 levels in breast cancer cells leads to uncontrolled cell proliferation and invasion by elevating the expression of Ezh2 [10]. Recently, miR-214 has been found to enhance the sensitivity of breast cancer cells to endocrine therapy (Tamoxifen and fulvestrant) through inhibition of autophagy by targeting p53.
UCP2 [11]. Nevertheless, it remains to be explored whether miR-214 expression is required for the chemotherapy sensitivity and, if so, how miR-214 regulates the drug sensitivity of breast cancer cells.

As a critically important tumor suppressor, p53 is activated by a variety of genotoxic agents, e.g., doxorubicin, leading to cell cycle arrest and cell death [12]. RFWD2, also known as COP1 an E3 ubiquitin ligase, is one of crucial antagonists of p53 [13]. RFWD2 is significantly overexpressed in breast cancer tissues and directly binds to and degrades p53, as thus eventually promoting tumor growth, particularly for those sustaining wild type p53 [14]. In this study, we revealed that miR-214 is down-regulated in breast cancer cells and tissues and its expression is correlated with longer disease free survival. Restoration of miR-214 in MCF7 cells leads to increased apoptosis and sensitivity to doxorubicin. In addition, RFWD2 is identified as a miR-214 direct target gene and ectopic expression of RFWD2 compromises miR-214-mediated apoptosis and cell growth arrest. Altogether, we demonstrate that miR-214, as a potential target for the development of cancer therapy, can activate p53 pathway by directly inhibiting RFWD2.

2. Materials and methods

2.1 Clinical samples

The experimental procedures were approved by the Ethics Committee at Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology. All breast cancer tissues and corresponding adjacent tissues were collected from 31 patients during 2008 and 2015. Among these 31 patients, 24 patients received doxorubicin treatment and were divided
into two groups, doxorubicin sensitive (n=15) and resistant patients (n=9), according to the efficacy assessments results after at least two cycles chemotherapy treatments. Written informed consent was obtained for all patient samples.

**Plasmids and miRNA oligonucleotides**

The RFWD2-Flag-expressing plasmid was purchased from Origene (Beijing, China). The 3’UTR of RFWD2 sequence was amplified by RT-PCR with TaKaRa Ex Taq (Takara) using following primers: 5’-AAGGTGCTAGAATTGGTAAGGG-3’ and 5’-CAACTGTGGCTCAATAAAACTTTATTTG-3’. Subsequently, the amplified fragment was cloned into the Sac I and HindIII sites of pMIR-REPORT Vector (Thermo Scientific) according to the manufacturer’s protocol. RFWD2-mutant-3’UTR was generated from the wild-type construct using the QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA). MiR-214 mimics and negative control (miR-NC) were synthesized by Genepharm (Shanghai, China).

**Cell culture and transient transfection**

Human breast cancer cell lines MCF7, MDA-MB-157, MDA-MB-468 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum, 50 U/ml penicillin and 0.1 mg/ml streptomycin. All cells were maintained at 37 °C in a 5% CO₂ humidified atmosphere. Cells seeded on the plate overnight were transfected with plasmids or miR-214 mimics using Lipofectamine 2000 transfection reagent following the manufacturer’s protocol (Thermo Scientific).

**Quantitative real time PCR analyses**

Total RNA was isolated from cells or tissues using Trizol following the manufacturer’s
protocol (Invitrogen, Carlsbad, CA, USA). RT-PCR for mature miR-214 was performed by using SYBR Premix ExTaqTM (Takara, USA) as previously described[15]. The RNA input was normalized to the human U6 snRNA. Relative expression values from three independent experiments were calculated following the $2^{-\Delta\Delta Ct}$ method.

Flow cytometry analyses

Cells transfected with miR-214 or miR-NC were fixed with ethanol overnight and stained in 500 µl of propidium iodide (Sigma-Aldrich) stain buffer (50 µg/ml PI, 200 µg/ml RNase A, 0.1% Triton X-100 in phosphate-buffered saline) at 37 °C for 30 min. The cells were then analyzed for DNA content using a BD Biosciences FACScan flow cytometer (BD Biosciences, San Jose, CA, USA). Data were analyzed using the CellQuest (BD Biosciences) and Modfit (Verity, Topsham, ME, USA) software programs.

Cell viability assay

To assess cell survival after doxorubicin treatment, the Cell Counting Kit-8 (CCK-8) (Dojindo Molecular Technologies, Rockville, MD, USA) was used according to the manufacturer’s instructions. Cells were seeded in 96-well culture plates (5000/100 µl per well) at 12 h post-transfection, incubated overnight at 37°C, then treated with doxorubicin at serial dilutions. Cell viability was determined by adding WST-8 at a final concentration of 10% to each well, and the absorbance of the samples was measured at 450 nm using a Microplate Reader (Molecular Device, SpectraMax M5e, Sunnyvale, CA, USA) after 3 hours of incubation at 37°C.

Immunoblotting

Protein sample preparation and immunoblotting were performed as previously described[16].
Briefly, the appropriate amount of tissues were quickly taken from liquid nitrogen and transferred into a lysate-containing homogenizer, grinded sufficiently, and incubated for 30 minutes on ice. Cells were harvested and lysed in lysis buffer (50 mM Tris/HCl pH7.5, 0.5% Nonidet P-40 (NP-40), 1 mM EDTA, 150 mM NaCl, 1 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride, 10 µM pepstatin A and 1mM leupeptin). Equal amounts of clear cell lysate (20-60 µg) were used for immunoblotting (IB) analyses. The primary antibodies used for immunoblotting were anti-RFWD2 mouse monoclonal antibody (clone 1E4, Sigma-Aldrich), anti-Cleaved PARP (#9541, Cell Signaling Technology), anti-p53 (DO-1, Santa Cruz Biotechnology), anti-p21(CP74, Neomarkers, Fremont, CA, USA), anti-PUMA (H-136, Santa Cruz Biotechnology) and anti-β-actin (C4, Santa Cruz Biotechnology).

**Luciferase reporter assay**

MCF7 or MDA-MB-157 cells were plated in a 24-well plate overnight and then were co-transfected with pMIR-REPORT luciferase constructs and miR-214 mimics or miR-NC. Cells were lysed 24h post-transfection for measuring luciferase activity using the Dual-Luciferase Reporter Assay System according to the manufacturer’s protocol (Promega).

**Statistical analyses**

Statistical analyses were performed using the statistical package SPSS (v. 13.0) or GraphPad Prism 6.0 (GraphPad Software Inc.). Statistical significance was determined using Student t test or one way analysis of variance (ANOVA) as appropriate. The Spearman correlation test was used for evaluating the correlations between miR-214 expression levels and RFWD2 protein levels in breast cancer samples. Survival curve was generated using the
Kaplan-Meier method and assessed using Gehan-Breslow-Wilcoxon test. P < 0.05 was considered statistically significant.

3. Results

3.1 MiR-214 is significantly down-regulated in breast cancer cells and doxorubicin resistant breast cancer tissues

To explore whether miR-214 was aberrantly expressed in breast cancers, we first examined miR-214 levels in three breast cancer cell lines and three normal breast tissues. As shown in the Fig. 1A, miR-214 expression is remarkably decreased in all three cell lines compared to the normal breast tissues. Further detection of miR-214 expression in 31 paired clinical breast cancer tissues demonstrated that miR-214 was significantly reduced in tumor tissues compared with the corresponding normal tissues (Fig. 1B; P < 0.001). To investigate the correlation of miR-214 level and clinical outcome, we divided the 31 patients into two groups based on the median value of miR-214 expression levels. Kaplan-Meier survival curves showed that patients with high miR-214 levels manifested a much longer disease progress survival (P=0.0013; Fig. 1C). In clinical treatment of breast cancer, patients with doxorubicin resistant always result in a shorter disease progress survival [5]. Prompted by the above results, we then assessed whether miR-214 level was correlated with doxorubicin resistance of breast cancer cells. The clinical patients were divided into two groups, doxorubicin sensitive group (n=15) and doxorubicin resistant group (n=9), according to the outcomes after doxorubicin treatment. Notably, miR-214 level is much lower in doxorubicin resistant group compared to the doxorubicin sensitive group (Fig. 1D; P<0.001). Altogether,
these results indicate that miR-214 is down-regulated in breast cancer, particularly, in the
doxorubicin resistant breast cancer. This observation also suggests that miR-214 may act as a
tumor suppressor which is probably required for breast cancer cell sensitivity to doxorubicin.

3.2 miR-214 induces apoptosis and sensitizes MCF7, but not MDA-MB-157 cells to
doxorubicin

To investigate the role of miR-214 in apoptosis of breast cancer cells, MCF7 (sustaining wild
type p53) and MDA-MB-157 (without p53 expression) cells were transfected with negative
control (miR-NC) or miR-214 mimics followed by FACS analysis. It was found that ectopic
expressed miR-214 significantly increased apoptosis of MCF7, but not MDA-MB-157 cells,
under both normal culturing and doxorubicin treatment conditions (Fig. 2A and 2D). The
induced apoptosis was further confirmed by induction of cleaved PARP and PUMA
expression in MCF7 cells (Fig. 2B and 2E). Notably, PUMA is a pro-apoptotic target gene of
p53, this result also suggests that miR-214 may induces p53 activity. Next, we carried out a
set of cell survival assays by treating MCF7 and MDA-MB-157 cells with doxorubicin.
Compared with the control group, ectopic expressed miR-214 significantly sensitized MCF7
cells to doxorubicin, with IC₅₀ decreased from 19.92 nM to 7.62 nM (Fig. 2C), whereas
miR-214 barely affected the doxorubicin efficacy to MDA-MB-157 cells (Fig. 2F). Taken
together, these results reveal that miR-214 boosts chemotherapeutic drug efficiency in breast
cancer cells probably through a p53-dependent manner.

3.3 RFWD2 is a direct target of miR-214 in breast cancer cells
To test whether miR-214 regulates p53 activity, we used online bioinformatics tools, TargetScan[17] and miRDB[18,19], to analyze the possible target genes of miR-214. Intriguingly, we found RFWD2 (also also known as COP1), a negative regulator of p53, as a candidate target gene for further study. We first amplified and cloned RFWD2-wt-3’UTR and RFWD2-mut-3’UTR (Fig. 3A) to the report vector and performed the luciferase assay in MCF7 or MDA-MB-157 cells. As expected, the reporter assay revealed that miR-214 markedly reduced luciferase activity of the RFWD2-wt-3’UTR reporter, but not the RFWD2-mt-3’UTR reporter (Fig. 3B). The regulation of RFWD2 expression by miR-214 was further confirmed by the observation that protein level of RFWD2 was drastically repressed by miR-214 expression in MCF7 and MDA-MB-157 cells (Fig. 3C). Consistently, the protein expression of p53 and its target genes, PUMA and p21, was remarkably induced by miR-214 in MCF7 cells (Fig. 3C). In addition, knock-down of p53 significantly decreased doxorubicin-induced apoptosis in MCF7 cells as measured by FACS analysis and cleaved PARP and PUMA levels (Fig. S1A and S1B). We next explored the relationship between miR-214 and RFWD2 levels in 22 tissues from breast cancer patients. Spearman’s correlation analysis revealed a statistically significant inverse correlation between the levels of miR-214 and RFWD2 protein (r = −0.6669; P =0.0006) (Fig. 3D). Collectively, these results demonstrate that miR-214 inhibits RFWD2 expression through directly targeting its 3’UTR and, consequently, leads to p53 activation in breast cancer cells sustaining wild type p53.

3.4 Ectopic expression of RFWD2 attenuates miR-214-induced apoptosis and drug sensitivity
of MCF7 cells

Since RFWD2 is a direct target gene of miR-214, we then investigate whether overexpression of RFWD2 can abolish the effect of miR-214 in breast cancer cells. As expected, ectopic expression of RFWD2 reduced p53 protein levels and diminished miR-214-mediated apoptosis under both normal culturing and doxorubicin treatment conditions as measured by FACS analysis and cleaved PARP and PUMA levels (Fig. 4A and 4B). The cell survival assay also showed that RFWD2 impaired miR-214-induced drug sensitivity of MCF7 cells, as IC\textsubscript{50} increased from 7.56 nM to 17.24 nM (Fig. 4C). Altogether, these results demonstrate that miR-214 induces apoptosis and doxorubicin-sensitivity of breast cancer cells through regulating the RFWD2-p53 cascade.

DISCUSSION

In the study, we find that miR-214 expression is down-regulated in breast cancer cells and, particularly, in doxorubicin resistant breast cancer tissues, which implies the potential correlation of miR-214 with doxorubicin resistance in breast cancer cells. Additionally, we reveal that restoration of miR-214 in breast cancer cell lines elicits apoptosis and enhances the cancer cell sensitivity to doxorubicin. Interestingly, this effect was observed in MCF7 cells with wild type p53, but not in the p53 null MDA-MB-157 cells, suggesting a p53-dependent role of miR-214 in breast cancer. Furthermore, we indeed demonstrate that miR-214 induces p53 activity through down-regulating RFWD2. In sum, our findings uncover a novel role of miR-214 in regulating p53 activity and chemosensitivity in breast cancer.
Although no attention had been paid to the effect of miR-214 on the chemotherapy sensitivity in breast cancer, miR-214 was shown to be closely associated with chemotherapy resistance in other cancers. For example, miR-214 enhances cisplatin-induced cytotoxicity via downregulation of Bcl2l2 in cervical cancer [20], which is in line with our findings established in breast cancer. Surprisingly and interestingly, miR-214 has also been found to inhibit cisplatin-induced apoptosis and induce drug resistance in human ovarian cancer and tongue squamous cell carcinomas by targeting PTEN and enhancing the AKT pathway [21] [22]. Therefore, miR-214 displays contrasting behavior in different human cancers, suggesting that the cell context determines the function of miR-214 as an oncogene or tumor suppressor gene. In light of our study, p53 status could be one of the determinants that dictate the fate of miR-214. Given that p53 is highly mutated in human cancers and that the mutant p53 not only loses the tumor suppressive activity, but also have oncogenic “gain-of function”, miR-214 is no longer able to regulate the RFWD2-p53 cascade and activates p53 in the p53-mutated cancers.

As a master tumor suppressor gene, TP53 is one of the most frequently (>50%) mutated genes in the overall human cancers [23]. However, there are still about 80% of breast tumors harboring wild type p53, suggesting that activation of wild type p53 is a promising strategy to treat breast cancer [24]. Thus, our study also highlights the possibility that miR-214 could be a potential druggable target, as this microRNA re-activates p53 and induces p53-dependent drug sensitivity.

The E3 ligase RFWD2 is ubiquitously expressed and high RFWD2 expression has been characterized in breast adenocarcinomas, hepatocellular carcinoma and pancreatic cancer.
tissues [14,25,26]. We also observed the highly expressed RFWD2 in breast cancer samples, which is reversely correlated with miR-214 levels (Fig. 3D). In addition, miR-214 elicits apoptosis by depressing RFWD2 expression. However, we observed that the ectopic expression of RFWD2 did not completely abrogate miR-214-induced apoptosis (Fig. 4A and 4B), implying that other downstream targets might be also involved in the miR-214 function. For example, miR-214 can enhance apoptosis and chemotherapy sensitivity by targeting Bcl2l2 in cervical cancer [20] and CUG-BP1 in esophageal squamous cancer cells [25], which may also be true in breast cancer.

In conclusion, this study demonstrates that miR-214 is down-regulated in breast cancer and restoration of miR-214 facilitates doxorubicin-induced apoptosis by inhibiting its target gene RFWD2 and activating p53. These results indicate that low expression of miR-214 contributes to the chemotherapeutic drug resistance of breast cancer cells, particularly those with wild type p53, and suggest that miR-214 could potentially be used in anti-cancer therapy as an adjunct strategy.

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Conflict of interest
The authors declare that they have no conflict of interest.
References


Figure legends

**Fig.1.** miR-214 is down-regulated in breast cancer cell lines and clinical tissues. (A) The relative miR-214 expression in three normal tissues and breast cell lines were measured by quantitative PCR, U6 was used as an internal normalized control. All data are presented as the mean±SEM of triplicate experiments(* p<0.01, two-tailed t-test, compared to normal samples). (B) Expression of miR-214 in 31 pairs of breast cancer specimens and adjacent normal breast tissues. * p<0.001, two-tailed t-test. (C) Kaplan-Meier analysis of disease free survival based on miR-214 levels in 31 breast cancer patients (Gehan-Breslow-Wilcoxon test, p=0.0013). (D) Comparison of miR-214 expression in clinical samples of doxorubicin sensitive (n=15) and resistant patients (n=9). * p<0.001, two-tailed t-test.
**Fig.2.** miR-214 promotes apoptosis and enhances doxorubicin sensitivity of MCF7, but not MDA-MB-157 cells. MCF7 (A-B) or MDA-MB-157(D-E) cells were transfected with miR-NC or miR-214 mimic. Cells were harvested 72h post-transfection for flow cytometry analysis (A, D) or immunoblotting with indicated antibodies (B, E). Quantification of Sub-G1 population is shown in (A, D). MCF7 (C) or MDA-MB-157 (F) cells were transfected with miR-NC or miR-214 and seeded in 96-well plates next day. Doxorubicin was supplemented for 72h before cell viability detection by CCK-8 as described in the Materials and methods. All data are presented as the mean±SEM of triplicate experiments (*p<0.01, two-tailed t-test in A).

**Fig.3.** miR-214 directly targets RFWD2 and thus activates p53 in breast cancer cells. (A) The binding site of miR-214 in the RFWD2 3’UTR (ENST00000367669.3). (B) The miR-214 mimic or negative control, a pMIR-REPORT vector containing the wild or mutant type of RFWD2 3’UTR, were co-transfected into MCF7 or MDA-MB-157 cells, and the relative firefly luciferase activity was measured 24h post-transfection. The data are presented as the mean±SD of three independent experiments (*p < 0.05, two-tailed t-test). (C) Immunoblotting analysis of RFWD2, p53, PUMA and p21 in breast cancer cells transfected with miR-214 mimic or negative control. (D) Correlation analysis of RFWD2 protein and miR-214 expression in clinical breast cancer samples (n=22, p=0.0006, two-tailed Pearson's R Test). The RFWD2 protein levels were detected by immunoblotting, and miR-214 expression was measured by qRT-PCR.
Fig. 4. Overexpression of RFWD2 attenuates miR-214-induced apoptosis and doxorubicin sensitivity in MCF7 cells. (A and B) MCF7 cells were co-transfected with RFWD2-Flag plasmid and miR-NC or miR-214 mimic. Cells were harvested 72h post-transfection for flow cytometry analysis (A) or immunoblotting with indicated antibodies (B). Quantification of Sub-G1 population is shown in (A). All data are presented as the mean±SEM of triplicate experiments (*p<0.01, two-tailed t-test). (C) MCF7 cells were co-transfected with RFWD2-Flag plasmid and miR-NC or miR-214 mimic and then seeded in 96-well plates next day. Doxorubicin was supplemented for 72h before cell viability detection by CCK-8 as described in Materials and methods.
Figure lists

**Fig.1.** miR-214 is down-regulated in breast cancer cell lines and clinical tissues.

**Fig.2.** miR-214 promotes apoptosis and enhances doxorubicin sensitivity of MCF7, but not MDA-MB-157 cells.

**Fig.3.** miR-214 directly targets RFWD2 and thus activates p53 in breast cancer cells.

**Fig.4.** Overexpression of RFWD2 attenuates miR-214-induced apoptosis and doxorubicin sensitivity in MCF7 cells.
Fig. 1

A

B

C

D

Relative miR-214 expression

Adjacent normal Breast cancer

Disease Free Survival

0 20 40 60 80 100 120

Months after operation

mRNA low

mRNA high

p=0.0013

Relative miR-214 expression

Dox-sensitive Dox-resistant

p=0.0013
Fig. 2

(A) MCF7

(B) MCF7

(C) MCF7

(D) MDA-MB-157

(E) MDA-MB-157

(F) MDA-MB-157

Apoptosis (Sub G1 %)

Dox Control

miR-Nc miR-214

Dox Control

miR-214 + -

cleaved PARP ~
PUMA ~
β-actin ~

Relative cell viability

Concentration of Dox (μM)

miR-NC miR-214

Relative cell viability

Concentration of Dox (μM)

miR-NC miR-214
**Fig. 3**

A

miR-214: 3’ UGACGGACAGACAC–GGACGACA 5’
RFWD2-3’UTR: 5’ ...CAAUUGUACUUGAUGGUGUGA... 3’
RFWD2-Mut-3’UTR: 5’ ...CAAUUGUACUUGAUGGACGACA... 3’

B

![Graph showing relative luciferase activity](image)

C

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D

![Graph showing miR-214 expression vs RFWD2 protein level](image)
Fig. 4

A

B

C

(A) Apoptosis (Sub G1 %)

- miR-NC
- miR-214
- miR-214+RFWD2

Control | Dox
---|---

(B) Western Blot

- miR-214
- RFWD2-Flag
- cleaved PARP
- PUMA
- RFWD2
- p53
- β-actin

Control | Dox
---|---
+
+
+
+
+
+
+
+
+

(C) Relative cell viability

- miR-NC
- miR-214
- miR-214+RFWD2

Concentration of Dox (µM)

0.001 | 0.01 | 0.1 | 1
---|---|---|---

0 | 20 | 40 | 60
---|---|---|---

100 | 80 | 60 | 40
---|---|---|---

120 | 100 | 80 | 60
---|---|---|---
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