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MiR-214 negatively regulates proliferation and WNT/β-catenin signaling in breast cancer

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Abstract. – OBJECTIVE: To analyze the function and mechanism of miR-214 in regulating breast cancer cell proliferation.

MATERIALS AND METHODS: MiR-214 expression level was measured by quantitative reverse transcription Polymerase Chain Reaction (qRT-PCR). The protein level of β-catenin, PCNA and WNT targets (cyclin D1 and c-Myc) was evaluated by Western blot analysis. The effects of miR-214 on cell proliferation and cisplatin sensitivity were assessed by Cell Counting Kit-8 (CCK-8) assay and/or 5-bromo-2'-deoxyuridine (BrdU) assay. The effect of miR-214 on β-catenin and WNT signaling activity was tested by luciferase reporter assay.

RESULTS: MiR-214 was significantly downregulated in breast cancer tissues and was inversely correlated with β-catenin expression. Forced expression of miR-214 in breast cancer cell line MCF-7 led to a decrease in cell proliferation and an increase in cisplatin sensitivity. Moreover, forced expression of miR-214 decreased the activity of WNT luciferase reporter and the luciferase reporter containing the 3'-untranslated region (3'UTR) of β-catenin, whereas antisense inhibitor of miR-214 showed an opposite effect. Finally, miR-214 decreased the expression of β-catenin and multiple WNT target genes.

CONCLUSIONS: MiR-214 is downregulated and serves as a novel tumor suppressor in breast cancer. Forced expression of miR-214 in breast cancer cells diminished cancer cell survival possibly through inhibiting WNT signaling by direct repression of β-catenin.

Key Words:
Breast cancer, WNT signaling, β-catenin, miR-214, Proliferation.

Introduction

Breast cancer is one of the major health threats of women worldwide, and its incidence is rapidly increasing among Chinese women[1]. Identifying the molecular mechanism for cancer development and progression would aid in developing better genetic therapeutic approaches for breast cancer.

MicroRNAs are small RNA single strands that exist in the cytoplasm, and it serves as a class of negative regulator for gene expression[2]. MicroRNAs preferentially bind to the 3'-untranslated region (3'UTR) of certain mRNAs through base pairing, leading to mRNA breakdown or the blockage of the protein translation process[2,3]. MicroRNAs are well conserved among species and some may play essential roles in regulating basic biological processes including cell death, cell proliferation and cell differentiation[4-6]. A large number of microRNAs have been identified to be abnormally expressed in breast cancer and are associated with clinical-pathological features, suggesting the critical role of microRNAs in controlling breast cancer pathogenesis[7,8]. Previous studies[9-12] demonstrated that miR-214 acted as a tumor suppressive microRNA in multiple cancers such as ovarian cancer, esophageal cancer, renal carcinoma, and rhabdomyosarcoma. However, conflict reports are still found in the existing literature, miR-214 induced pro-tumoral effects in pancreatic cancer[13], and it promotes metastasis in lung adenocarcinoma[14]. The role of miR-214 in breast cancer is also enigmatic, both the tumor-suppressive and the promoting role have been reported[15,16]. Moreover, the lack of understanding of the molecular mechanism of miR-214 in breast cancer underlies the importance for further investigating the function of miR-214 in breast cancer.

WNT signaling is one of the essential signal transduction pathways in the carcinogenesis of multiple cancers[17,18]. Once the WNT ligand binds to the Frizzled receptor, β-catenin is able to accumulate and translocate to nucleus and functions as the co-activator of TCF/LEF transcriptional factor, leading to the transactivation of downstream genes. Previous studies[19,20] have shown that β-catenin mediated ca-

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nonical WNT signaling is essential for breast cancer cell proliferation, and cancer stem cell maintenance, suggesting that targeting WNT signaling might be feasible in breast cancer treatment.

In the present work, we determined the expression of miR-214 in breast cancer tissues and tested the potential role of miR-214 in regulating breast cancer cell proliferation and drug sensitivity. Importantly, we evaluated the functional association between miR-214 and WNT/β-catenin signaling; Western blot and luciferase reporter assay identified β-catenin as the direct target of miR-214. Our study demonstrated the tumor-suppressive role of miR-214 in breast cancer, and these effects may be a result of its inhibitory action on the WNT/β-catenin signaling.

Materials and Methods

Human Tissue Samples

To compare the expression level of miR-214 in breast cancer tissues and normal tissues, 20 pairs of matched tumor tissues and adjacent normal tissues were used. The tissues were collected during the period from August 2014 to April 2015. Samples were snap frozen in liquid nitrogen and stored at -70 °C. All the patients were fully informed of the use of the samples for scientific research.

Cell Culture

Human breast cancer cell line MCF-7 was obtained from the Cell bank of Chinese Academy of Sciences (Shanghai, China). Cells were cultured using Dulbecco's Modified Eagle Medium (DMEM, Hyclone, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS, Hyclone) in a CO_2 incubator at a constant temperature of 37°C. The atmosphere in the incubator was kept humidified, and the concentration of CO_2 is 5%.

Transfection

A negative control, miR-214 mimics and antisense strand (AS-214) were all purchased from Genepharma (Shanghai, China), and they were transfected into MCF-7 cells using Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, CA, USA) according to the instructions.

Western Blot

The protein was extracted from the human tissues or the cell samples using RIPA lysis buffer (Beyotime, Shanghai, China), after quantification by a BCA kit, the same amount of protein was loaded onto sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels. The Western blot analyses were performed following a previously described procedure[21]. Nitrocellulose membranes were incubated with primary antibodies against β-catenin (Cell Signaling Technology, Danvers, MA, USA), c-Myc (Cell Signaling Technology, Danvers, MA, USA), PCNA (Cell Signaling Technology, Danvers, MA, USA), cyclin D1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and β-actin (Shanghai, China). Horseradish Peroxidase (HRP)-linked secondary antibodies and ECL substrate were from Pierce Biotechnology (Rockford, IL, USA).

Quantitative Polymerase Chain Reaction (qPCR)

The total RNA of tissue samples and treated cells were extracted with miRNeasy Mini Kit (Qiagen, Crawley, UK), reverse transcribed using the PrimeScript-RT reagent kit (Takara Co., Ltd., Otsu, Shiga, Japan), and amplified on a real-time thermocycler (ABI 7500 Real-time PCR system, Applied Biosystems, Foster City, CA, USA). The primer sets for miR-214 reverse transcription and amplification were purchased from RiboBio, U6 was used for normalization and the data were processed with the $2^{-\Delta\Delta Ct}$ method. The experiments were performed in triplicate.

Cell Survival Assay

Cell survival was analyzed using a cell counting kit-8 (CCK-8) method. The kit was purchased from Beyotime biotechnology and the experiments were performed according to the manufacturer's protocol. For the evaluation of the cisplatin sensitivity, the inhibition rate was calculated and plotted. Each treatment was performed for five times.

5-Bromo-2-deoxyUridine (BrdU) Assay

After transfection of miR-214 mimics or negative control, BrdU proliferation assay was performed using a kit purchased from Millipore (Billerica, MA, USA). The OD450nm was tested and normalized to that of a negative control group. The experiment was performed in triplicate.

Luciferase Reporter Assay

The TOPFLASH luciferase plasmid was used to detect WNT activity, and we also constructed pMIR-REPORT-CTNNB1-3'UTR plasmid by inserting the fragment in the 3'UTR of β-catenin containing the miR-214 putative binding region into the pMIR-REPORT plasmid (AMBION, Au-

Figure 1. Differential expression of miR-214 and β-catenin in human breast cancer tissue samples and adjacent normal tissue samples. ***A,*** Expression of miR-214 in breast cancer tissues (BC) and adjacent normal tissues (N). ***B-C,*** Western blot image (B) and statistic quantification ***(C)*** of the expression of β-catenin in BC and N. $^{*}p<0.05$ vs. N, n=20.

stin, TX, USA). For Luciferase assay, the reporter plasmid (TOPFLASH or pMIR-REPORT-CTNNB1-3'UTR), pRL-TK (Promega, Madison, WI, USA) and microRNAs (miR-214, AS-214 or negative control) were cotransfected for 48h. A Dual Luciferase detection system (Promega) was then used for luciferase activity detection.

Statistical Analysis

Data were presented as means ± SEM, and t-test was used to analyze the significance between 2 groups. One way Analysis Of Variance (ANOVA) was used for the statistics in luciferase reporter assays, Dunnett's t-test was used for post hoc pairwise comparison. A two tailed *p*-value less than 0.05 was considered statistically significant.

Results

Differential Expression of miR-214 and β-catenin in Human Breast Cancer Tissue Samples and Adjacent Normal Tissue Samples

20 pairs of breast cancer tissue samples and the adjacent normal tissue samples were collected, and miR-214 expression was then analyzed by qPCR. We detected a significant decrease in miR-214 expression in breast cancer tissues compared with the adjacent tissues (Figure 1A). As WNT signaling is important for cell proliferation in breast cancer cells, we analyzed the expression of its important component, β-catenin. We found that β-catenin was increased in breast cancer tissues (Figure 1B and C). This inverse relationship between miR-214 and β-catenin indicates that miR-214 may be important for the control of WNT signaling dependent cell proliferation.

MiR-214 Inhibits Proliferation of Human Breast Cancer Cells

To better understand the function of miR-214, we overexpressed miR-214 in human breast cancer cells MCF-7. We found a sharp decrease of cell proliferation after miR-214 overexpression, as shown by the CCK-8 assay (Figure 2A) and BrdU assay (Figure 2B). Importantly, the nuclear proliferation marker, PCNA, was markedly decreased (Figure 2C and D), which is inconsistent with the above results. These data support the tumor suppressive role of miR-214 in breast cancer.

MiR-214 Increases Cisplatin Sensitivity of Human Breast Cancer Cells

We also analyzed whether miR-214 can have potential clinical application to increase chemotherapeutic drug sensitivity of breast cancer cells. Cell survival analysis by CCK-8 assay revealed that cells overexpressing miR-214 are more sensitive to cisplatin-induced cell death, as exemplified by a significantly higher inhibitory rate at multiple cisplatin concentrations (Figure 3).

MiR-214 Inhibits Canonical WNT Signaling via Direct Downregulation of β-Catenin

We next explored the potential mechanism of the tumor suppressive role of miR-214. Since we detected an inverse relationship between miR-214 and β-catenin and the online database predicted their direct binding (Figure 4A), we speculated that miR-214 might influence cell proliferation by modulating WNT signaling activity. We constructed luciferase reporter containing the 3'UTR of β-catenin, results showed that miR-214 inhibited while the antisense inhibitor of miR-214 increased the luciferase activity (Figure 4B). We then transfected a WNT (TOPFLASH) reporter

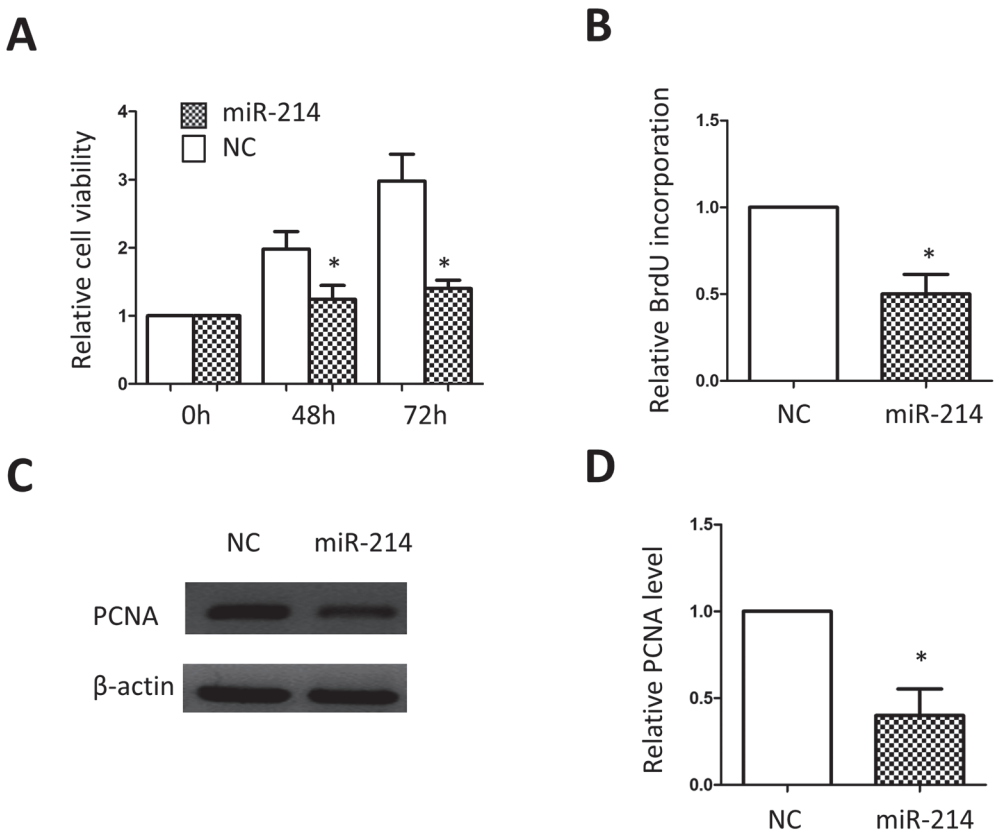

Figure 2. MiR-214 inhibits proliferation of human breast cancer cells. ***A,*** Relative cell viability of MCF-7 cells transfected with miR-214 or negative control (NC) at 0h, 48h and 72h, $^{*}p<0.05$ vs. NC at each time point, n=5. ***B,*** Cell proliferation measured by BrdU assay in cells transfected with miR-214 or NC at 48h, $^{*}p<0.05$ vs. NC, n=3. ***C,*** Western blot image© and quantification ***(D)*** of PCNA expression in in cells transfected with miR-214 or NC at 48h, $^{*}p<0.05$ vs. NC, n=3.

into MCF-7 cells, and similar results were obtained (Figure 4C), suggesting that miR-214 inhibits WNT signaling activity through direct repression of β-catenin in human breast cancer cells. More importantly, after miR-214 overexpression, the protein level of β-catenin was significantly decreased, and two WNT transcriptional targets, c-Myc and cyclin D1, were decreased concurrently (Figure 4D-G). All these data suggested that miR-214 is a negative regulator of the canonical WNT signaling, which is essential for breast cancer cell proliferation; downregulation of miR-214 might contribute to an increased WNT signaling, leading to an increased cell proliferation.

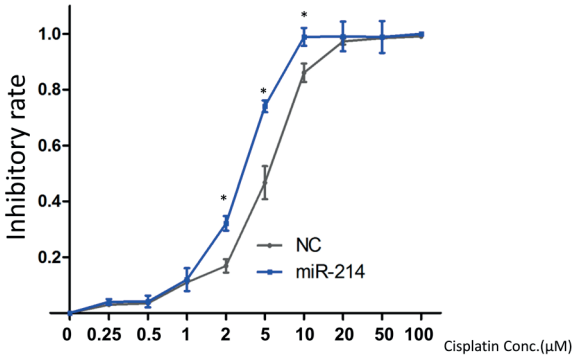

Figure 3. MiR-214 increases cisplatin sensitivity of human breast cancer cells. The inhibitory rates of various doses of cisplatin at 48h in cells transfected with miR-214 or NC. $^{*}p<0.05$ vs. NC at each cisplatin concentration, n=5.

Discussion

Aberrantly regulated cell proliferation is one of the key reasons of tumorigenesis. The WNT signaling pathway, which plays important roles in regulating cell proliferation and differentiation during embryonic development, has been shown to be hyperactivated in multiple cancers, particularly in breast cancer[22-24]. Understanding the regulatory mechanism of WNT signaling would be helpful to

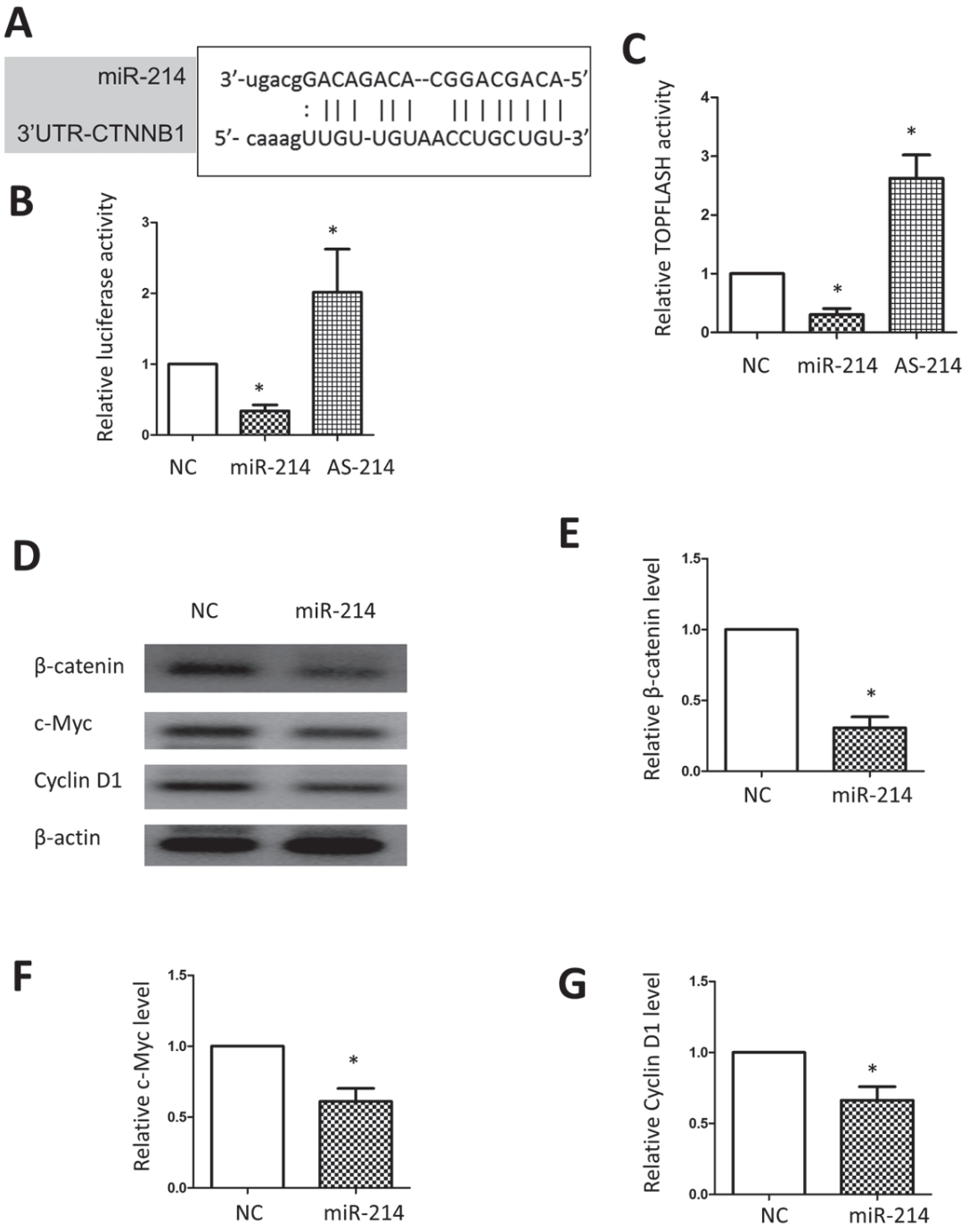

Figure 4. MiR-214 inhibits canonical WNT signaling via direct downregulation of β-catenin. ***A,*** The schematic binding between miR-214 and β-catenin 3'UTR. ***B-C,*** The relative luciferase activity of pmiR-REPORT-CTNNB1-3'UTR (B) and TOPFLASH ***(C)*** in cells transfected with miR-214 or antisense inhibitor of miR-214 (AS-214). ***D-G,*** The Western blot images (D) and quantification ***(E, F*** and ***G)*** of β-catenin and WNT targets c-Myc and cyclin D1 of MCF-7 cells transfected with NC and miR-214 for 48h, $^{*}p<0.05$ vs. NC, n=3.

develop therapeutic approaches to suppress the proliferative activity of breast cancer cells.

The emerging evidence of post-transcriptional regulation of gene expression by microRNAs in breast cancer implied that microRNAs may be key players in tumorigenesis and thus might be utilized to molecular or gene therapy. The role of several microRNAs in breast cancers has been

identified, and most of which are implicated in the basic biologic processes such as proliferation, cell death, and trans-differentiation[8]. The function of miR-214 in breast cancer has been rarely reported. Wang et al[16] initially identified the promoting effect of miR-214 on breast cancer cell invasiveness, and upregulation of miR-214 in tumor tissues was detected in their study. Conversely, Liu et al[15] reported the downregulation of miR-214 in breast cancer tissues along with negative association between miR-214 expression level and Ki67 index[15], and Yu et al[25] also suggested that miR-214 increased the anticancer efficacy of tamoxifen and fulvestrant. In the current paper, we observed a significant decrease of miR-214 expression in cancer tissues compared with adjacent normal tissues. We also found that miR-214 expression is negatively associated with the expression level of β-catenin, a critical component of the canonical WNT signaling pathway. Functional assays revealed that miR-214 inhibited cell proliferation and increased the sensitivity of breast cancer cells to undergo cisplatin-induced cell death. Our work together with the study by Liu et al[15] shows that miR-214 is a tumor suppressor in breast cancer.

Accumulating evidence has shown that WNT signaling pathway could be a potential therapeutic target for breast cancer[24]. The pleiotropic oncogenic role of WNT signaling has highlighted its essential role in breast cancer progression: canonical WNT signaling promotes proliferation and maintains stem-like cells[20,26], and WNT signaling is required for epithelial to mesenchyme transdifferentiation[26,27]. WNT pathway inhibitors such as pyrvinium pamoate and CWP232228 exhibited multiple anticancer effects[28-30], suggesting that targeting WNT signaling seems to be an effective way to overcome breast cancer. Notably, previous researches uncovered multiple microRNAs to be critical regulators of the WNT signaling activity in breast cancer. For example, miR-142 targets APC that is an essential for the degradation of β-catenin, which causes the activation of canonical WNT signaling[31]; miR-374 also functions as an activator of WNT signaling by repressing WIF1, PTEN, and WNT5A that are critical for β-catenin activation[32]. In our current study, since bioinformatics analysis supported the potential binding between miR-214 andβ-catenin mRNA, we speculated whether miR-214 could also be an important regulator of WNT/β-catenin signaling after we have observed the negative relation between the expression of β-catenin and miR-214 in clinical samples. Forced expression of miR-214 evidently inhibited β-catenin and the two WNT transcriptional targets (Cyclin D1 and C-myc) *in vitro*. Particularly, the TOPFLASH luciferase activity, which is the readout of the activity of WNT signaling, was dramatically suppressed by miR-214. Luciferase assay of the 3'UTR region further validated that the predicted miR-214 targeting site in β-catenin mRNA is authentic. Intriguingly, miR-214 also targets β-catenin during skin development and esophagus cancer progression, our results is consistent with these reports and firstly implicated a similar function of miR-214 in the regulation WNT signaling in breast cancer.

It should be noted that as the targets of microRNAs may not be unique, we are not able to specify whether other signaling pathways might also get involved in miR-214 induced growth inhibition. Nonetheless, our data demonstrated the critical involvement of WNT/β-catenin signaling in the anti-proliferation and cisplatin-sensitizing function of miR-214 in breast cancer and suggested that targeting WNT/β-catenin by microRNAs might represent a potential therapeutic method. Since all the data were obtained from *in vitro* experiments, more *in vivo* data are still needed to confirm this mechanism. Nevertheless, our study provided a foundation for the future investigation of the functions of miR-214 in breast cancer as a critical regulator of WNT signaling.

Conclusions

We have found that miR-214 is downregulated in breast cancer; it functions as a tumor suppressor and is a key regulator to suppress WNT/β-catenin signaling. Our study unraveled the molecular mechanism that controls WNT regulated oncogenicity, and may provide implications for therapeutic intervention to increase drug sensitivity and to control the proliferation of breast cancer cells.

Conflict of interest

The authors declare no conflicts of interest.

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