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Original Article

miR-30a inhibits breast cancer progression through the Wnt/β-catenin pathway

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Abstract: Background: miR-30a is a microRNA associated with the progression of malignant tumors such as gastric cancer, colon cancer, prostate cancer, and lung cancer, and can regulate the proliferation and migration of breast cancer (BC) cells in vitro. However, its expression, function, clinical significance and relationship with the Wnt/β-catenin pathway in human BC were still unclear. Methods: Immunohistochemistry, Western blotting and real-time quantitative PCR (RT-qPCR) were used to measure the expressions of miR-30a and β-catenin in 114 pairs of human BC tumor tissues and adjacent normal tissues which were collected from March 2014 to October 2015. The effect of miR-30a on the expression of β-catenin was studied in the MCF-7 cells in vitro. Results: The expression levels of miR-30a in human BC tumor tissues were significantly lower than they were in the adjacent normal tissues ($P < 0.001$), and significantly higher in β-catenin protein ($P < 0.001$), but there was no significant different in β-catenin mRNA ($P = 0.3816$). The immunohistochemistry results showed that β-catenin protein was only expressed on the cell membrane in paracancerous normal tissues, but β-catenin protein was expressed on the cell membrane and cytoplasm in BC tumor cells. In addition, there was a significantly negative correlation ($r = -0.816$, $P < 0.001$) between the expression miR-30a and β-catenin protein in BC tissues. The age of onset, PR expression, ER expression, and HER-2 expression of the BC patients were not related to miR-30a or β-catenin protein expression ($P > 0.05$). Tumor diameter, histological grade, lymph node metastasis, TNM stage, and the prognosis of BC patients ($P < 0.05$) were significantly related to miR-30a or β-catenin protein expression. In MCF-7 cells, miR-30a regulated the accumulation of β-catenin protein by inhibiting the expression of BCL9 in BC cells. Conclusion: miR-30a was lowly expressed in breast cancer tissues and highly in β-catenin protein, and miR-30a might block the Wnt/β-catenin pathway by inhibiting the accumulation of β-catenin, and then inhibiting breast cancer progression.

Keywords: miR-30a, breast cancer, progression, β-catenin

Introduction

Since 2010, the morbidity and mortality of cancer in the Chinese have been rising. According to the latest data released by the National Cancer Center, the new cancer growth rate in China is 3%, accounting for 1/4 of new cancer cases worldwide [1]. According to the cancer statistics of China in 2015 [2], the five most common cancers among women were breast cancer (BC), lung and bronchial cancer, stomach cancer, colorectal cancer and esophageal cancer, and of these 15% were breast cancer.

microRNA is a small non-coding RNA that plays an important role in regulating the transcription of genes and can regulate cell proliferation, differentiation, apoptosis, metabolism, and invasion/migration activities by targeting their target gene [3, 4]. Previous studies have shown that miRNAs play an important role in the regulation of the progression of many malignancies [5, 6], such as prostate cancer [7, 8], gastric carcinoma [9], colorectal cancer [10], and breast cancer [11], etc. More importantly, dysregulated miRNAs in tumor tissues have been used as targets for the development of therapeutic cancer drugs [12, 13]. miR-30a was found to be involved in the progression of malignant tumors such as gastric cancer [14], colon cancer [15], prostate cancer [16], and lung cancer [17] and could regulate the proliferation and migration of breast cancer (BC) cells in vitro [18]. However, the expression of miR-30a in BC tissues was still unclear.

Table 1. Relationship between miR-30a or β-catenin and the clinical pathology of BC patients

Items	miR-30a expression		χ^2	P	β-catenin expression		χ^2	P
	High (n = 61)	Low (n = 53)			High (n = 55)	Low (n = 59)		
Age (years)								
≤ 50	23	21	0.044	0.834	22	22	0.255	0.614
> 50	38	32			33	27		
Tumor diameter (cm)								
≤ 2	23	10	4.893	0.027	8	25	10.717	0.001
> 2	38	43			47	34		
ER								
Positive	25	23	0.068	0.795	20	28	1.437	0.231
Negative	36	30			35	31		
PR								
Positive	33	23	1.300	0.254	23	33	2.269	0.132
Negative	28	30			32	26		
HER-2								
Positive	21	25	1.913	0.167	18	28	2.566	0.109
Negative	40	253*0.48			37	31		
Histological grade								
I	30	10	27.078	< 0.001	9	31	19.732	< 0.001
II	26	16			18	24		
III	5	27			28	4		
Lymph node metastasis								
Yes	36	45	9.242	0.002	49	32	16.812	< 0.001
No	25	8			6	27		
TNM stage								
I	24	9	10.256	0.006	11	22	14.178	0.001
II	21	16			13	24		
III	16	28			31	13		

β-Catenin is a key molecule in the Wnt signaling pathway, and many human malignancies have been found to be associated with abnormally activated Wnt/β-catenin pathways [19, 20]. In addition, miR-30a was found to affect the activation of the Wnt/β-catenin pathway by regulating BCL9 expression in myeloma cells [21] or PRDM1 in glioma cells [22]. However, the relationship between miR-30a and the Wnt/β-catenin pathway in breast cancer has not been confirmed. In this paper, we measured the expression of miR-30a and β-catenin in BC tissues and studied their regulatory relationship in MCF-7 cells.

Materials and methods

Tissue and statement

114 breast cancer patients (all female) were selected as subjects for this study, which was conducted from January 2014 to December 2015 in Changzhou Central Hospital. The patients' tumor tissue and adjacent normal tissues were removed surgically and collected. The tissues were divided into three parts, one for making paraffin sections, and the other two were stored in liquid nitrogen. The ages of the 114 breast cancer patients ranged from 42 to 71 years, and 63 was the median age. Other clinical data is shown in **Table 1**. In addition, all subjects (or their guardians) included in this study consented to the research protocol and signed an informed consent form. The ethics committee of Changzhou Central Hospital approved this research protocol.

Real-time quantitative PCR

Trizol was used to extract the total RNA of the tissues or cells. The extracted RNA was reverse transcribed into cDNA using a PrimeScript™RT

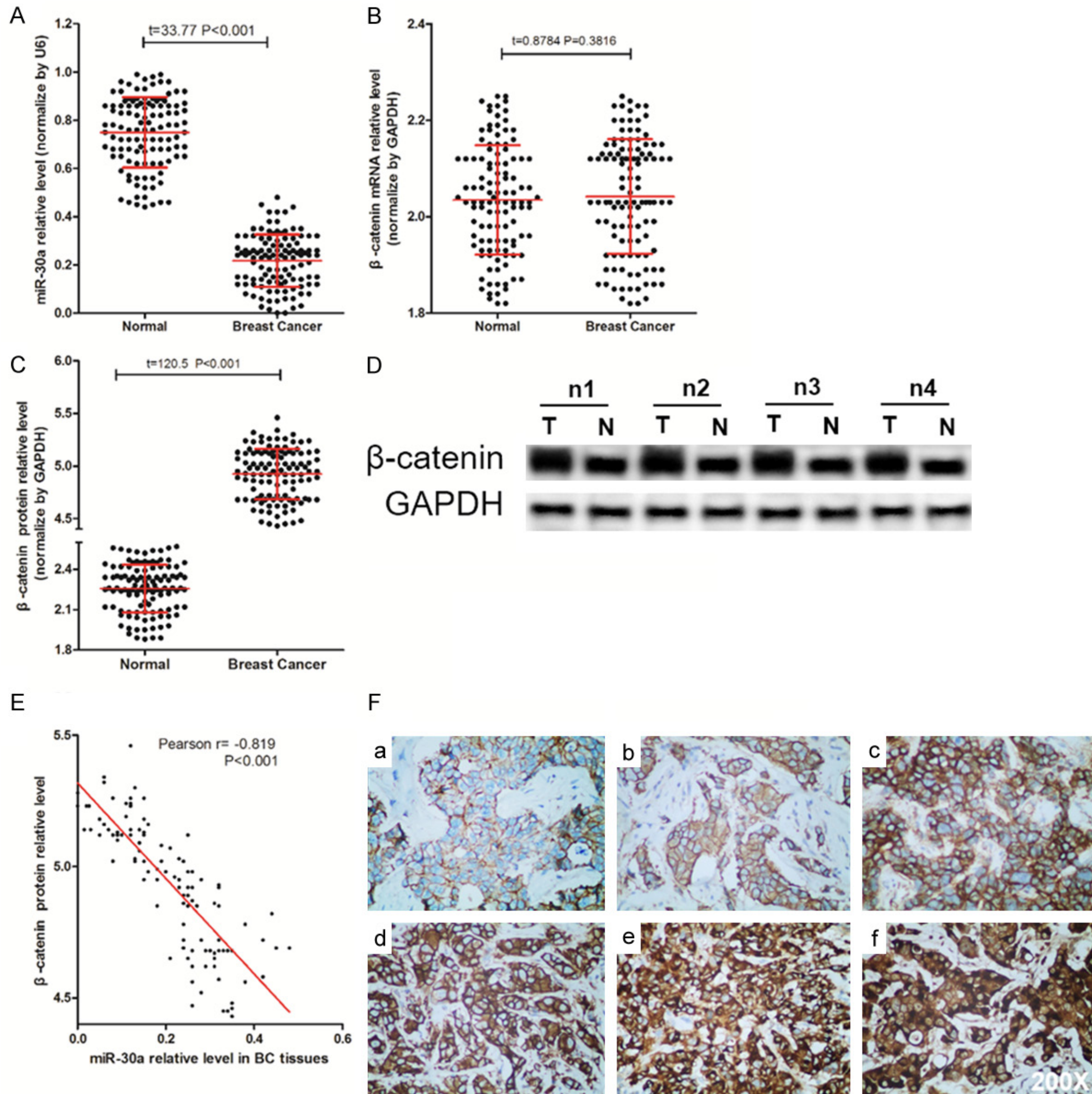

Figure 1. Expression of miR-30a and β-catenin. (A, B) RT-qPCR was used to detect the expression of miR-30a (A) and β-catenin mRNA (B) in BC tissues and paracancerous normal tissues; (C) Expression of β-catenin protein was counted in BC tissues and paracancerous normal tissues; (D) T = neuroblastoma tissues, N = paracancerous normal tissues, nx = number x of patient with BC; (E) The expression miR-30a and β-catenin protein in the BC tissues. (Fa) β-catenin protein was only expressed on the cell membranes in paracancerous normal tissues; (Fb) Expression of β-catenin protein in BC tissues; (Fc) β-catenin protein was only expressed on the cell membranes in BC tissues (-); (Fd) β-catenin protein was expressed on the cell membranes and weak positive expression on the cytoplasms and cytoplasms in BC tissues (+); (Fe) β-catenin protein was expressed on the cell membranes and positive expression on the cytoplasms and the cytoplasms in BC tissues (++); (Ff) β-catenin protein was expressed on the cell membranes and a strong positive expression on the cytoplasms and the cytoplasms in BC tissues (+++); Normal = paracancerous normal tissues.

Master Mix reverse transcription kit (RR036B, Takara, Beijing, China). The PCR parameters were set at: 37°C/60 minutes, 85°C/5 seconds. A 20 μl real-time fluorescence quantitative PCR (RT-qPCR) system was prepared according to the SYBR Green qPCR Master Mix kit instructions (638320, TakaRa, Beijing, China) and amplified using ABI 7500 fluorescence quantitative PCR instrument (Applied Biosystems, Maryland, USA). The PCR parameters were set at: 95°C/30 s, [90°C/5 s, 65°C/30 s]-40 cycles. The PCR primers were: miR-30a-F, 5'-ACACTCCAGCTGGGTGTAAACATC-CTCGAC-3', miR-30a-R, 5'-TGGTGTCGTGGAGT-

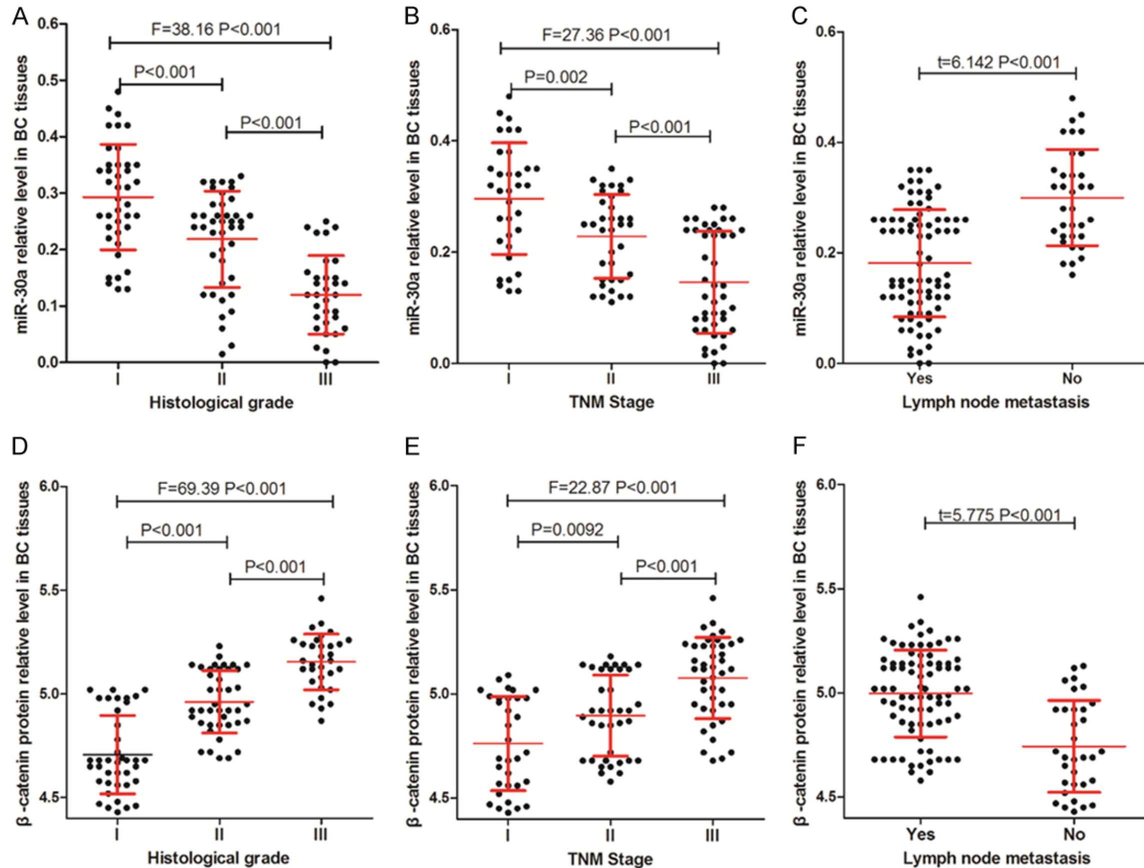

Figure 2. Expressions of miR-30a and β-catenin protein in different breast cancer tissues. (A-C) Comparison of miR-30a expression in different histological grades (A), TNM stage (B) and lymph node metastasis (C) in breast cancer tissues; (D-F) Comparison of β-catenin protein expression in different histological grades (D), TNM stage (E) and lymph node metastasis (F) in breast cancer tissues.

CG-3'; U6-F, 5'-CTCGCTTCGGCAGCACA-3', U6-R, 5'-AACGCTTCACGAATTTGCGT-3'; BCL9-F, 5'-GGCCATACCCCTAAAGCACTC-3', BCL9-R, 5'-CGGAAATACTTCGCTCCCTTTT-3'; β-catenin-F, 5'-AAAGCGGCTGTTAGTCACTCG-3', β-catenin-R, 5'-CGAGTCATTGCATACTGTCCAT-3'; GAPDH-F, 5'-CTGGGCTACACTGAGCACC-3', GAPDH-F, 5'-AAGTGGTCGTTGAGGGCAATG-3'.

Western blot

Tissue or cell lysates were separated by SDS-page and then transferred to a PVDF membrane. Primary antibody: anti-BCL9 (1:500, ab37305, Abcam, Cambridge, UK), or anti-β-catenin (ab32572, 1:5000, Abcam, Cambridge, UK), or anti-GAPDH (ab9484, 1:3000, Abcam, Cambridge, UK). Second antibody: goat anti-rabbit (ab150077, 1:1000, Abcam, Cambridge, UK), or goat anti-rat (ab150117, 1:1000, Abcam, Cambridge, UK). The primary antibody was incubated overnight at 4°C and the second antibody was incubated for 1 hour at room temperature.

Immunohistochemistry

Immunohistochemistry was used to measure the β-catenin protein expression of the BC tissues or the adjacent normal tissues using a VECTASTAIN® Elite® ABC Kit (Vector Laboratories, Boston, MA, USA). A β-catenin antibody (ab32572, 1:5000, Abcam, Cambridge, UK) was used as a primary antibody (PBS instead of primary antibody as a negative control) and incubated overnight at 4°C. Goat Anti-Rabbit IgG H & L (HRP) (1:1000, Abcam, Cambridge, UK) was incubated as a secondary antibody at 37°C for 2 h. Finally, 5 fields per slice were photographed and evaluated: no color was negative (-), a faint yellow was weak positive (+), yellow was positive (++), claybank or tan was

Table 2. Univariate and multivariate Cox regression analysis for the prognosis of BC patients

Clinical parameters	Univariate analysis			Multivariate analysis		
	OR	95% CI	P	OR	95% CI	P
Age	1.245	0.417-3.658	0.624			0.586
Tumor diameter	2.029	0.809-4.627	0.083			0.823
ER positive	3.256	1.241-5.324	0.069			0.162
PR positive	3.164	0.986-4.238	0.062			0.159
HER-2 positive	3.342	0.429-04.824	0.041			0.084
Histological grade	3.514	0.912-11.254	0.028	2.739	1.127-12.414	0.031
Lymph node metastasis	3.435	1.020-5.184	< 0.001	2.685	1.432-9.867	0.024
TNM stage	4.022	1.615-11.231	0.003	2.612	1.004-10.628	0.047
MiR-30a expression	6.545	2.318-18.210	< 0.001	6.028	3.042-15.917	< 0.001
β-catenin expression	5.488	3.218-13.657	< 0.001	5.124	2.987-12.368	0.002

Note: OR = odds ratio, 95% CI = 95% confidence interval; SPSS statistical analysis software was used for multivariate Cox regression analysis. When *P* was less than 0.05, the results did not show the OR and 95% CI.

strongly positive (+++). Two pathology deputy chief physicians who were blinded to the patients' diagnoses evaluated the stained tissue.

Cell and cell transfection

MCF-7 cells (HTB-22, ATCC, VA, USA), from the human breast adenocarcinoma cell line were cultured with DMEM medium (12491-15, ThermoFisher, CA, USA), to which was added 10% fetal bovine serum (10100-147, ThermoFisher, CA, USA) and 1% penicillin-streptomycin (156-40055, ThermoFisher, CA, USA). miR-30a-NC, the miR-30a-mimic, and the miR-30a-inhibitor were designed and synthesized by Shenggong Bioengineering Co., Ltd. (Shanghai, China), and were directly transferred into the cells using the Lipofectamine™ 2000 transfection reagent (11668019, Invitrogen, CA, USA). For the wild type or the mutation mRNA 3'-UTR of BCL9, they were first connected to pisCHECK2 (Promega, WI, USA) and then were transfected into the cells as miRNA.

Statistical analysis

The data were analyzed by SPSS 20.0 for statistical analysis and were stated as the mean ± standard deviation or the frequency. Student's *t*-test or a chi-square test was used to compare the differences between two groups. Comparisons between multiple groups were performed using the single factor ANVOA method, and the Duncan method was used for post hoc testing. The correlations between two groups was analyzed by Pearson. The Cox regression model was used to test the univariate and multivariate analyses for the survival times of the BC patients. The survival curves of the BC patients based on the miR-30a or β-catenin protein expressions were drawn by the Kaplan-Meier method. A log-rank test was used to compare the differences of the survival curves. $P < 0.05$ indicated a significant difference.

Results

miR-30a and β-catenin expression in BC tissues

The expressions of miR-30a and β-catenin were measured in 114 pairs of breast cancer (BC) tissues and paracancerous normal tissues, and we found that the expression of miR-30a in the BC tissues was significantly lower than it was in the paracancerous normal tissues ($P < 0.001$) (**Figure 1A**), and there was a significantly negative correlation ($r = -0.816$, $P < 0.001$) between the expression miR-30a and β-catenin protein in the BC tissues (**Figure 1E**).

Although there was no significant difference between the expression of β-catenin in the BC tissues and the paracancerous normal tissues ($P = 0.3861$) (**Figure 1B**), the expression of β-catenin protein in the BC tissues was significantly higher than it was in the paracancerous normal tissues ($P < 0.001$) (**Figure 1C**, **1D**). In addition, immunohistochemistry was used to localize the expression of β-catenin in the BC tissues and paracancerous normal tissues, and we found that β-catenin protein was only expressed on the cell membranes in the para-

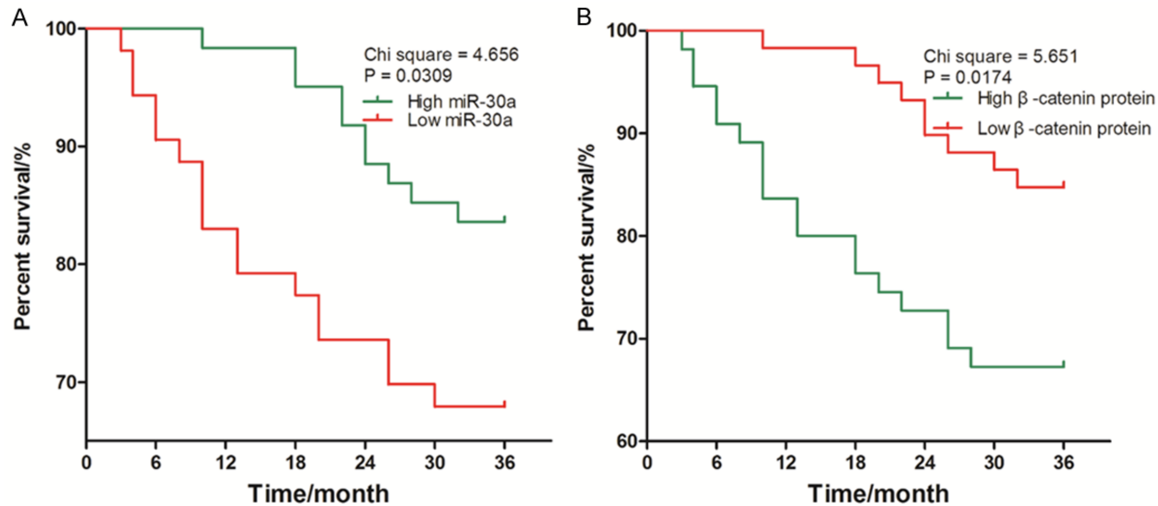

Figure 3. Survival curves of BC patients with different miR-30a or β-catenin protein expressions.

cancerous normal tissues, but β-catenin protein was expressed on the cell membranes and cytoplasms in the BC tissues. This indicates that β-catenin was deposited from the cell membranes into the cytoplasms and nuclei.

The relationship between miR-30a or β-catenin and the clinical pathology of BC patients

According to the expression levels of miR-30a or the β-catenin protein in breast cancer tissues, tissues from 114 breast cancer patients were divided into two groups, and the miR-30a low expression group was the expression level of miR-30a < the median value of 114 BC patients, and the same as β-catenin protein. We analyzed the relationship between miR-30a or β-catenin protein and the clinical pathology of BC patients. The results showed that the age of onset, PR expression, ER expression, and HER-2 expression of the BC patients were not related to miR-30a or β-catenin protein expression ($P > 0.05$). Tumor diameter, histological grade, lymph node metastasis, and TNM stage of BC patients ($P < 0.05$) were significantly related to miR-30a or β-catenin protein expression (**Table 1**). With the increase of histological grade or TNM stage in BC patients, the expression of miR-30a in the BC tissues was gradually decreased (**Figure 2A**, **2B**), but the expression of β-catenin protein in the BC tissues was gradually increased (**Figure 2D**, **2E**). In addition, the expression of miR-30a in the BC tissues with lymph node metastasis was significantly lower than that in BC tissues without lymph node metastasis ($P < 0.001$) (**Figure 2C**), but the expression trend of β-catenin protein was just the opposite (**Figure 2F**).

The effect of miR-30a or β-catenin expression on the prognosis of BC patients

114 BC patients were followed up at least once every four months or the patients came to hospital for a review. The factors influencing the survival times of BC patients were analyzed using the COX regression model. The results showed that (**Table 2**) the miR-30a expression level (OR = 6.028, 95% CI = 3.042-15.917) and the β-catenin protein expression level (OR = 5.124, 95% CI = 2.987-12.368) were independent risk factors influencing the prognosis of BC patients. Postoperative survival in BC patients with low expression of miR-30a was significantly lower than it was for patients with high expression of miR-30a ($P = 0.0309$) (**Figure 3A**), and postoperative survival in BC patients with high expression of β-catenin protein was significantly lower than it was in patients with low expression of β-catenin protein ($P = 0.0174$) (**Figure 3B**).

miR-30a knockdown promoted the accumulation of β-catenin protein in BC cells

In other cancer studies, such as those looking at multiple myeloma and prostate cancer, miR-30a has been found to regulate the Wnt/β-catenin signaling pathway by targeting BCL9 protein expression. We searched for binding

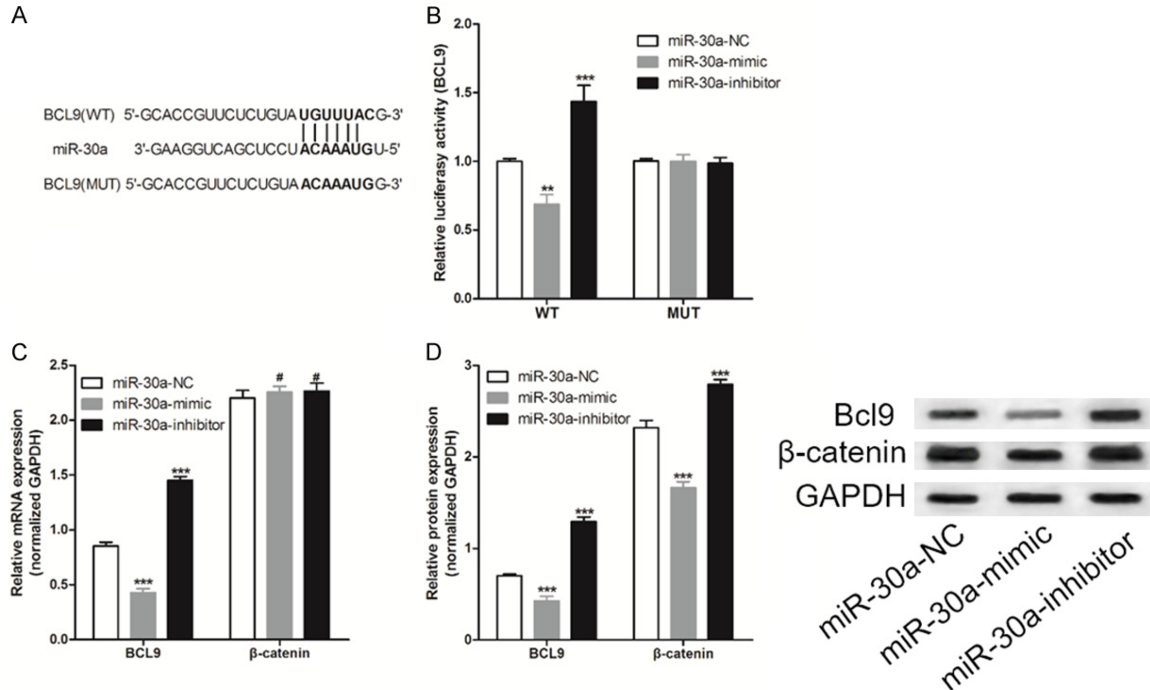

Figure 4. miR-30a regulated β-catenin protein accumulation by targeting BCL9. (A) WT-BCL9 3'UTR luciferase reporter vector, and a MUT-BCL9 3'UTR luciferase reporter vector with mutations on miR-30a binding sites of the BCL9 3'UTR was constructed; (B) miR-30a-NC/miR-30a-mimic/miR-30a-inhibitor were transected into MCF-7 cells, and luciferase activity was measured; (C) RT-qPCR was used to measure the expressions of BCL9 and β-catenin mRNA in different MCF-7 cells; (D) Western blot was used to detect the expression of BCL9 and β-catenin protein in different MCF-7 cells; 3 independent repetitions per experiment; ** was P < 0.01, *** was P < 0.001 and # was P > 0.05 vs miR-30a-NC group.

sites for miR-30a and BCL9 from the starBase database. To confirm that miR-30a could regulate BCL9 expression by binding to the BCL9 3'-UTR end, we used the luciferase gene reporter system. The results showed that transfection of miR-30a-mimics significantly decreased WT type 3'-UTR luciferase activity (P < 0.001), and the miR-30a-inhibitor significantly increased in MCF-7 cells. But it didn't work in MUT. Further, as shown in **Figure 4C**, **4D**, the miR-30a-inhibitor can increase the expression BCL9 mRNA and protein, but it only increases the expression of β-catenin protein and does not work with β-catenin mRNA. This means that miR-30a knockdown promotes the accumulation of β-catenin protein in BC cells.

Discussion

miR-30a is located at the 6q13 position on human chromosomes. Although it does not encode any protein, it plays an important role in many human diseases [23, 24], especially in cancer [25]. Previous research has shown that miR-30a not only negatively regulates the TGF-β1-induced epithelial-mesenchymal transition and peritoneal fibrosis by targeting Snai1 [26], but also functions as a tumor suppressor and novel therapeutic tool in many malignant tumors, such as gastric cancer [14], colon cancer [15], prostate cancer [16], and lung cancer [17]. In this paper, we found that the expression of miR-30a in 114 samples of BC tissues was significantly lower than it was in adjacent para-cancerous normal tissues, and was related to the tumor diameter, histological grade, lymph node metastasis, TNM stage, and the prognosis of BC patients. It means that miR-30a plays an important role in the occurrence and development of breast cancer.

TNM staging, lymph node metastasis and histological grading of the cancer are macroscopic representations of tumorigenesis and development, and their intrinsic nature is inseparable from the biological characteristics of tumor cell growth, proliferation, apoptosis and migration, and even more in-depth molecular mecha-

nisms may be closely related to multiple factors such as oncogene activation, tumor suppressor gene mutation inactivation, and abnormal changes in multiple signaling pathways [27, 28]. In this study, the expression of miR-30a in 114 samples of BC tissues decreased with the increase of TNM staging and tissue grade and lymph node metastasis. In addition, many previous studies had reported that miR-30a inhibited the proliferation, migration, and other biological functions of breast cancer cells in vitro by targeting Eya2 [18] and Notch1 [29], or via the p53/miR-30a/ZEB2 axis [30] and the miR-30a-5p/UBE3C axis [31]. Therefore, whether it is in breast cancer or in other malignant tumors, miR-30a exhibits the function of a tumor suppressor gene.

Because the development of tumors is the result of long-term interaction of multiple oncogenes and tumor suppressor genes through multiple signaling pathways, a tumor suppressor gene or oncogene may participate in the growth, proliferation and migration of tumor cells through multiple signaling pathways [32, 33]. The Wnt/β-catenin pathway is highly conserved during biological evolution, and participates in the regulation of cell growth, development and differentiation, and is regulated closely at the level of transcription and post-transcriptional modification [34], and its abnormal activation has been confirmed with the occurrence of various tumors including breast cancer [35, 36]. More and more studies have shown that the Wnt/β-catenin pathway plays an important role in the development and progression of breast cancer [37], invasion and metastasis [38, 39], and chemotherapy drug resistance [35], and many miRNAs play a key role in it, such as miR-34a [40] and miR-100 [41].

Although the relationship between miR-30a and the Wnt/β-catenin pathway in breast cancer has not been confirmed, miR-30a was found to affect the activation of the Wnt/β-catenin pathway by regulating BCL9 expression in myeloma cells [21] or PRDM1 in glioma cells [22]. In this study, we also found that there was a significantly negative correlation between the expression miR-30a and β-catenin protein in BC tissues, and miR-30a could regulate the accumulation of β-catenin protein in BC cells in vitro. β-catenin has been found to play a key role in the regulation of the biological characteristics of stem cells at various stages of breast development, and the activation or aggregation of the β-catenin signaling pathway leads to abnormal cell growth, differentiation, metabolism and biology, and eventually to breast cancer [42, 43]. β-Catenin is a key molecule in the Wnt/β-Catenin signaling pathway and plays a dual role in the signal transduction of the Wnt/β-Catenin signaling pathway: it can be used as a marker of pathway activation and can also bind to E-cadherin on the membrane to regulate cell-to-cell interaction and adhesion, affecting the aggressiveness of cancer cells [44]. In normal cells, β-catenin is localized in the cell membrane and is used to bind to E-cadherin on the membrane to form a complex and the content in the cytoplasm remains low or even nil, and a small amount of unbound β-catenin in cytoplasm is degraded by the APC/GSK-3β/Axin complex. However, when the Wnt pathway is abnormally activated, the degradation process of β-catenin is inhibited, allowing it to accumulate in the cytoplasm and enter into the nucleus to form a complex with TCF/LEF, which promotes the transcriptional activity of the target gene downstream of the pathway, resulting in an increase in transcriptional activity and abnormal biological characteristics in cells.

All in all, miR-30a was lowly expressed in breast cancer tissues and highly expressed in β-catenin protein, and miR-30a might block the Wnt/β-catenin pathway by inhibiting the accumulation of β-catenin, and then inhibiting breast cancer progression.

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Disclosure of conflict of interest

None.

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