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MicroRNA-421 inhibits caspase-10 expression and promotes breast cancer progression

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Breast cancer is one of the most prevalent and fatal diseases around the world. The mechanism of tumorigenesis in breast cancer remains to be clarified. miR-421 plays an oncogenic role in many cancers. Although, the clinical significance of miR-421 in patients with breast cancer is still to be investigated. Caspase-10 is one of the initiator of apoptosis. But the relationship between miR-421 and caspase-10 has not been investigated. In the present study, we found that miR-421 was expressed much higher in breast cancer tissues compared to those in adjacent non-tumor tissues. Furthermore, miR-421 promotes cell proliferation and colony formation *in vitro*. miR-421 inhibits cell apoptosis probably through restraining caspase-10 expression. Thus, miR-421 might be a potential diagnostic maker and therapeutic target for breast cancer.

Key words: miRNA-421, caspase-10, apoptosis, breast cancer

With more than one million newly diagnosed patients every year, breast cancer continues to be one of the most prevalent diseases around the world [1]. However, the mechanism of tumorigenesis in breast cancer remains to be clarified. Though huge advancements have been achieved in prolonging the survival of patients with breast cancer, most patients could not escape the fate of tumor recurrence after surgery. Thus, elucidating the molecular mechanism of oncogenesis and finding precise makers for target therapy for breast cancer is necessary, as well as urgent.

MicroRNAs (miRNAs) are short, non-coding RNA molecules that regulate gene expression at transcriptional or post-transcriptional levels [2] to adjust the signaling pathways in various cellular processes. Recently, the roles of miRNAs in many kinds of human malignancies have been investigated, as well as in breast cancer [3, 4]. Among the myriad of identified miRNAs, miR-421 intrigues the scientists. miR-421 plays an oncogenic role in many cancers. Compared with those in corresponding non-tumor tissue, miRNA-421 was found to be highly expressed in tissue from hepatocellular carcinoma [5], osteosarcoma [6], lung adenocarcinoma [7], gastric cancer [8], and pancreatic cancer [9]. Furthermore, in patients with hepatocellular carcinoma [5], osteosarcoma [6], or gastric cancer [8], high expression levels of miR-421 were associated with poor prognosis. However, the clinical significance of miR-421 in patients with breast cancer is still to be investigated.

Apoptosis is a process of programmed cell death that sustains balance in the number of cells in organisms. Insufficient apoptosis results in uncontrolled cell proliferation, and sometimes leads to cancer. Caspase-10, encoded by CASP10 gene, is considered as one of the initiator in the hierarchy of apoptotic cascade [10, 11]. It has been reported that miR-421 inhibits the expression of caspase-3, which is downstream of caspase-10 in the apoptosis process [10]. But the relationship between miR-421 and caspase-10 has not been investigated.

In the present study, we found that miR-421 was expressed much higher in breast cancer tissues compared to adjacent non-tumor tissues, and miR-421 promotes cell proliferation and colony formation *in vitro*. miR-421 inhibits cell apoptosis probably through restraining caspase-10 expression. Thus, the results indicate that miR-421 may be a potential diagnostic maker and a therapeutic target for breast cancer.

Patients and methods

Patients and tissue samples. A total of 65 pairs of specimens (paired tumor and adjacent non-tumor tissues) of primary breast cancer were obtained by surgical resection at the Department of Breast and Thyroid Surgery, Affiliated

Zhongshan Hospital of Dalian University between January 2010 and December 2014. The specimens were immediately frozen in liquid nitrogen until analysis. Related important clinical information, including patients' age, tumor size, histological grade, oestrogen receptor, progesterone receptor, HER-2, and Ki-67 status, were carefully reviewed. The study was approved by the Institutional Review Board of Dalian Medical University and informed consent was obtained from each patient.

RNA isolation and Quantitative real-time PCR. Total RNA was extracted from tissues using the Trizol reagent (Invitrogen, Carlsbad, CA). The miR-421 expression level was determined by quantitative real-time RT-PCR using Taqman assay kits (Applied Biosystems, Foster City, CA), with the forward primer as 5'-ATCAACAGACATTAATTGGGCGC-3' [12]. U6 small nuclear RNA was used as internal normalized reference, with the primers as 5'-CGCTTCGGCAGCACATATACTAA-3' (forward) and 5'-TATGGAACGCTTCACGAATTTGC-3' (reverse). To quantify mRNA levels of CASP10 gene, reverse transcription PCR was performed using PrimeScript RT Reagent Kit with cDNA Eraser (Takara, Dalian, China), and quantitative real-time PCR was performed using SYBR Premix Ex Taq (Takara). The primer sequences of CASP10 were 5'-CTGAAATGACCTCCCTAAGT-3' (forward), and 5'-AGGAGGTGTCACTATCTGGAT-3' (reverse) [13]. GAPDH gene was used as an internal control, with the primers as 5'-ACACCCACTCCTCCACCTTT-3' (forward) and 5'-TTACTCCTTGGAGGCCATGT-3' (reverse). Gene expression was quantified using the 2–ΔΔCT method, and normalized to the control.

Immunohistochemistry staining. IHC staining was performed as previously described [14]. In short, tumor tissues were dewaxed and rehydrated before conducting antigen retrieval. Slides were incubated with anti-Caspase-10 antibody (Abcam, Cambridge, USA) at 4 °C overnight, followed by incubation with an HRP-conjugated secondary antibody at room temperature for 1 hour. Diaminobenzidine (DAB) was used for coloration, and dark brown was considered to be positive. The strength of positivity was quantified by considering the percentage of positive cells and the staining intensity.

Cell lines and cell culture. Human breast cancer cell line MDA-MB-231 was obtained from the Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China). Cells were cultured in Leibovitz's L-15 medium (Gibco) containing 10% fetal bovine serum (Gibco) in a humid atmosphere at 37 °C with 5% CO_2.

Immunoblotting analysis. To analyze protein expression in cells, immunoblotting analysis was performed as previously reported [8]. Antibodies against Caspase-10 were obtained from Abcam (Cambridge, USA). β-actin (Sigma Aldrich, St. Louis, USA) was used as a loading control.

Lentivirus infection. The cell line construction was performed as previously reported [8]. The lentivirus carrying miR-421 and the corresponding negative control, an empty lentiviral vector, were obtained from Hanyin (Shanghai, China). MDA-MB-231 cell were infected with the miR-421-overexpressing lentivirus (231/miR-421) or the negative control lentivirus (231/NC). 48 h later, the infected cells were subjected to selection with 3 μg/ml puromycin.

Cell proliferation assay. The cell proliferation was measured by 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) Assay. 4×10^3 MDA-MB-231 cells in 200 μl medium were incubated in 96-well culture plates. Cell growth was detected using MTT reagent with Synergy H4 Hybrid Reader. Briefly, the culture medium was removed at day 1, 2, 3, 4, 5, 6 and 7, and 0.5 mg/ml MTT in 200 μl medium was added to each well and incubated for 4 h, followed by treatment with 150 μl of DMSO for 10 min. Data were collected by measuring the absorbance of optical density (OD) at 490 nm. The assays were repeated three times in triplicate.

Colony formation assay. For plate colony formation, 250 cells were seeded in 6-well plate. Triplicate cultures of each cell type were maintained at 37 °C in a 5% CO_2 atmosphere, and fresh medium was fed every 3 days. The number of colonies with >50 cells in each well was counted onday 14. The assays were repeated three times.

Cell apoptosis assay. Apoptosis was assessed by Annexin V-FITC (Invitrogen, Carlsbad, CA, USA) and flow cytometry. 1×10^6 cells were seeded in a 6-well plate and cultured for at least 48 hours. All the cells were collected and then washed twice with pre-chilled PBS, and resuspended in 1× binding buffer (1×10^6 cells/ml). Cells (100 μl) were stained with 5 μl annexin V-FITC and 5 μl propidium iodide (PI) for 15 min, and 400 μl 1× binding buffer was added. Analysis was conducted using a FC500 flow cytometer with CXP software (Beckman Coulter, Fullerton, CA, USA) within 1 h.

Statistical analysis. All the statistics were expressed as mean ± standard deviation (SD) of three independent experiments. The student's t-test was used to evaluate the between-group difference. The Mann-Whitney U test was used to analyze the relationship between the levels of miR-421 and the clinical features of the patients. Correlation analysis between relative expressions of miR-421 and CASP10 mRNA was done using a two-sided Spearman correlation analysis. SPSS v.16.0 (SPSS Inc., Chicago, IL) and GraphPad Prism 5.0 (GraphPad Software, La Jolla, CA) were used to conduct all the relative analyses. $P<0.05$ was set to be statistically significant.

Results

miR-421 is highly expressed in breast cancer tissue. To investigate the expression level of miR-421 in breast cancer, qRT-PCR was performed in frozen samples from pairs of breast cancer tissue and adjacent non-tumor tissue from 65 patients. The results showed that miR-421 expression levels in cancer tissues was higher than those in corresponding para-cancerous tissues, and the difference was statistically significant (Figure 1; **p<0.01). Interestingly, the higher expression level of miR-421 was significantly associated with larger

Table 1. Association between miR-421 level and clinicopathological characteristics in patients with breast cancer.

Clinicopathological features	n	miR-421 (mean±SD)	p-value
Age (years)			
≤50	34	1.03±1.14	0.306
>50	31	1.16±0.96	
Tumor size (cm)			
≤2	28	0.74±0.67	**0.020**
>2	37	1.36±1.21	
Histological grade			
I~II	29	0.90±0.72	0.078
III	19	1.69±1.53	
NA	17		
ER status			
Positive	38	1.11±0.96	0.319
Negative	24	1.05±1.26	
NA	3		
PR status			
Positive	27	1.16±1.07	0.439
Negative	35	1.02±1.10	
NA	3		
HER-2 status			
Positive	25	1.12±1.32	0.487
Negative	39	1.04±0.86	
NA	1		
Ki67 status			
≤50%	41	0.91±0.89	**0.036**
>50%	24	1.40±1.25	
NA			

All data were calculated using Mann-Whitney U test. p<0.05 is defined as statistically significant.

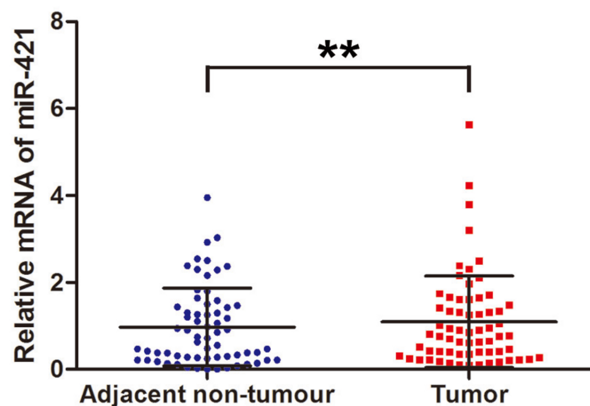

Figure 1. The expression of miR-421 is lower in breast cancer tissue than in adjacent non-tumor tissue. **p<0.01.

Table 2. Association between miR-421 mRNA level and caspase-10 protein status in breast cancer tissues.

Caspase-10 status	n	miR-421 (mean±SD)	p-value
Positive	36	0.85±0.76	**0.023**
Negative	23	1.58±1.34	
NA	6		

The data were calculated using Mann-Whitney U test. p<0.05 was set to be statistically significant.

sizes of tumor and higher expression levels of Ki-67 protein, but not with age, histological grade, hormone receptor status or HER-2 status (Table 1). The results indicate that miR-421 acts as onco-miRNA in breast cancer.

Overexpression of miR-421 promotes cell proliferation and colony formation. To further confirm whether miR-421 plays oncogenic role in breast cancer, we overexpressed the cDNAs coding miRNA-421 in breast cancer cell line MDA-MB-231.

The results from MTT assays showed that cells with high levels of miR-231 (231/miR-421) grew faster than corresponding control cells (231/NC) (Figure 2B). Furthermore, colony formation analysis showed that the number of colonies was twice as high in 231/miR-421 cells than in 231/NC cells (Figure 2C, D). The results suggest that amplification of miR-421 significantly promotes the proliferation ability of breast cancer cells *in vitro*.

miR-421 inversely regulates the expression of caspase-10. To clarify the relationship between miR-421 and caspase-10, we further examined the expression of both mRNA level and protein level of caspase-10 in the tissues from 65 cases of breast cancer. qRT-PCR results revealed that mRNA level of caspase-10 was in inverse correlation with miR-421 (Figure 3A; Spearman R = –0.799, p<0.01). Furthermore, immunohistochemistry assays indicated that tissue with less intensity staining of anti-caspase-10 antibody had higher levels of miR-421 (p<0.05, Figure 4, Table 2). Therefore, it is safe to conclude that both, the transcription level and translation level of caspase-10, were inversely related to the expression level of miR-421 in breast cancer tissue.

To further validate the relationship between miRNA-421 and caspase-10, we examined the expressions of both mRNA and protein of caspase-10 in MDA-MB-231 cells with or without ectopic expression of miR-421. The results again demonstrated that in 231/miR-421 cells, the mRNA level of caspase-10 was significantly lower than that in 231/NC cells (Figure 3B). Moreover, immunoblotting analysis showed that the protein level of caspase-10 was lower in cells overexpressing miRNA-421 than in the corresponding control cells (Figure 3C). The results further proved that miR-421 inversely regulated the expression of caspase-10 mRNA and protein.

Amplification of miR-421 inhibits cell apoptosis. To elucidate whether the relationship between miR-421 and caspase-10 further influences the apoptotic ability in breast cancer cells, we analyzed cell apoptosis by flow cytometry *in vitro*. The results showed that after overexpressing miR-421

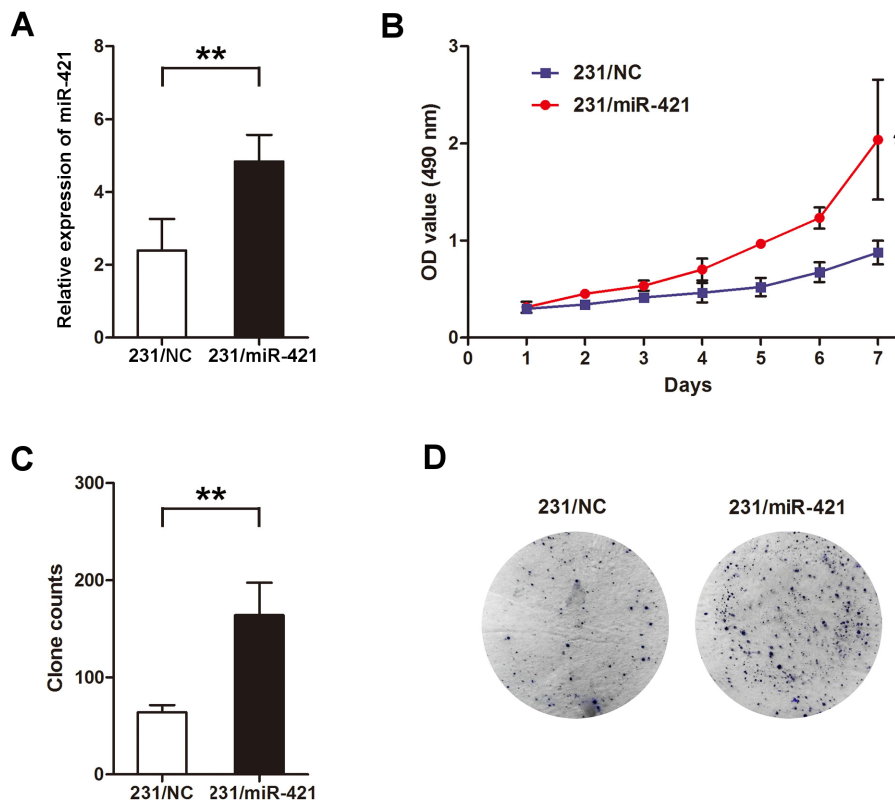

Figure 2. The effect of miR-421 on cell proliferation and colony formation. (A) qRT-PCR assays confirm the overexpression of miRNA-421 in MDA-MB-231 cells (231/miR-421) compared to corresponding control cells (231/NC). Alteration of cell growth curves (B) and plate colony formation (C) before and after overexpressing the miR-421. Data were collected from three independent experiments, and analyzed for statistic significance. Error bars = SD. **p<0.01, *p<0.05. (D) Representative images of plate colony formation.

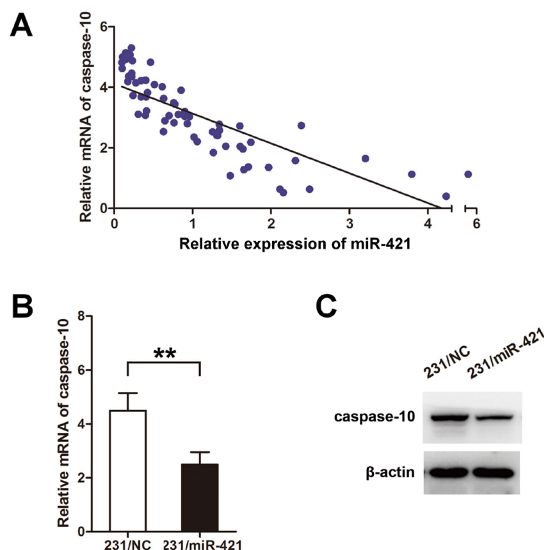

Figure 3. The relationship between miRNA-421 and caspase-10. (A) Spearman correlation analysis revealed the inverse correlation between miR-421 and caspase-10 mRNA levels ($R = -0.799$, $p<0.01$). (B) qRT-PCR showed that the mRNA level of caspase-10 in 231/miR-421 cells was lower than in 231/NC cells. (C) Immunoblotting analysis revealed that the protein level of caspase-10 was lower in 231/miR-421 than in 231/NC cells.

in MDA-MB-231 cells, the proportion of total apoptotic cells was significantly decreased in cells with ectopically expressed miR-421 (Figure 5A, C–D). Therefore, the percentage of early apoptotic cells was reduced by more than 70% (Figure 5B, C–D). The results suggest that amplification of miR-421 inhibits cell apoptosis in breast cancer cells *in vitro*, probably through caspase-10.

Discussion

The present study illustrates that miR-421 was higher expressed in breast cancer tissue than in adjacent non-tumor tissue, and miR-421 inversely regulated both the mRNA and protein levels of caspase-10, which might further inhibit cell apoptosis and promote cell proliferation to promote tumorigenesis in breast.

miR-421 has been regarded as an onco-microRNA in various types of cancers, including neuroblastoma [15, 16], gastric cancer [8], lung adenocarcinoma [7], pancreatic cancer [9], hepatocellular carcinoma [5, 9], and nasopharyngeal carcinoma [17], etc. It is reported that miR-421 was also upregulated in IBC compared to non-IBC [4]. But the role of miR-421 in breast cancer is still largely illusive. In neu-

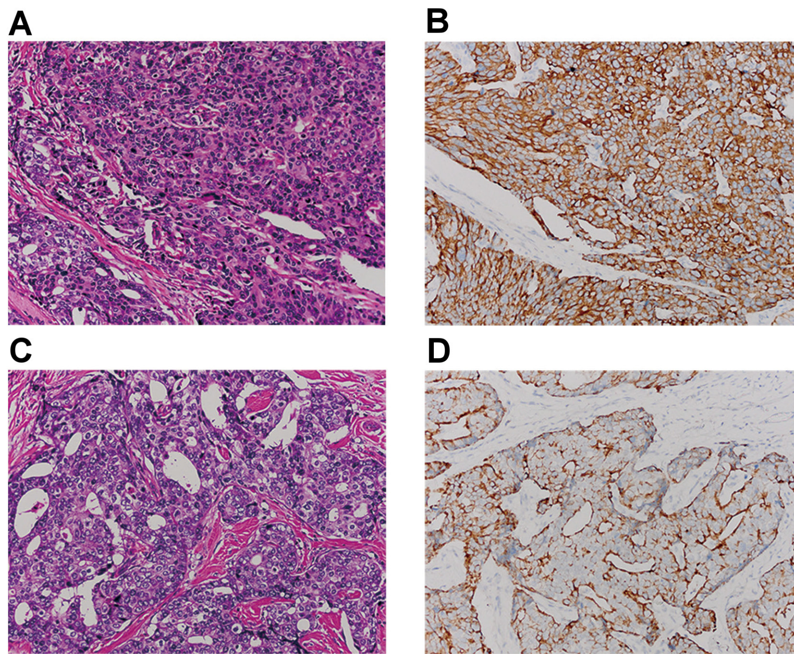

Figure 4. The expression of caspase-10 in breast cancer tissue. Representative images of strong staining (B) and weak staining (D) in immunohistochemistry anti-caspase-10 assays and the corresponding HE staining slides from the same tissue (A, B) ×200.

roblastoma cells, N-Myc-regulated miR-421 downregulated ATM, resulting in S-phase cell cycle checkpoint changes and an increased sensitivity to ionizing radiation [15]. In gastric cancer cells [15] and biliary tract cancer cells [15], miR-421 promotes cell proliferation and cell-cycle progression. However, the regulatory characteristics of miR-421 in breast cancer are still poorly understood. In the present study, we found that the expression of miR-421 was significantly increased in breast cancer tissues compared to para-carcinoma tissues. Furthermore, a higher expression level of miR-421 was significantly associated with larger tumors and higher index of Ki-67 protein. The results indicate that higher level of miR-421 might be linked to elevatedr aggressiveness of breast cancer.

Lerebours et al reported that higher miR-421 expression was related to a statistically significant poorer metastasis-free survival in non-IBC patients [4]. Due to limited time on follow-up in this study, there was not enough information to draw any conclusion on the influence of miR-421 on patient survival (data not shown). The data will be updated in following reports.

Moreover, *in vitro* studies indicate that in breast cancer cell line MDA-MB-231, overexpression of miR-421 significantly promotes cell proliferation and colony formation ability. Collectively, the results suggest that miR-421 acts as an oncogenic element in breast cancer both *in vivo* and *in vitro*.

Apoptosis is a vital mechanism keeping cells from excessive proliferation, and thereby it prevents tumorigenesis. In nasopharyngeal carcinoma cells, miR-421 suppresses apoptosis via inhibiting fork-head box protein O4 (FOXO4) signaling [17]. And in gastric cancer cells, miR-421 restrains apoptosis through caspase-3 [8; 17]. Herein, we found that high expression of miR-421 inhibited cell apoptosis in breast cancer cell line MDA-MB-231. In breast cancer cell

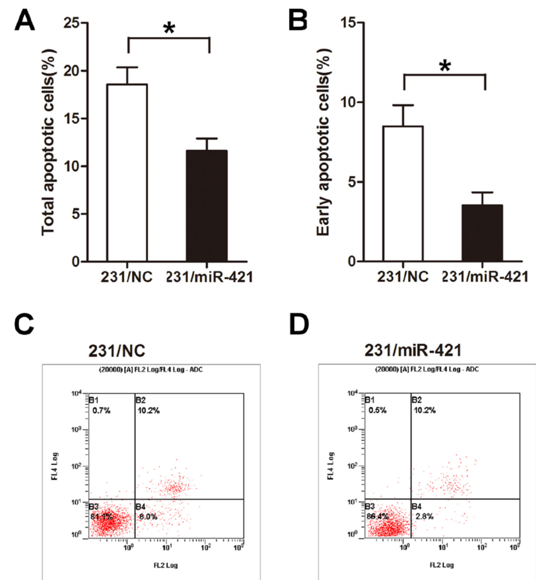

Figure 5. The effect of miR-421 on cell apoptosis. Quantitative analysis of proportion of the early (A) and total (B) apoptotic cells in MDA-MB-231 cells. Error bars = SD. *p<0.05. Representative images of flow cytometry analysis in control cells (231/NC) (C) and miR-421 overexpressing cells (231/miRNA-421) (D).

line MCF-7, it is reported that caspase-10 sensitized cells to TRAIL-induced apoptosis [18]. But the relationship between miR-421 and caspase-10 has not been investigated. In this study, we found that in breast cancer tissue, high expression of miR-421 was inversely correlated to both, the mRNA level and protein level of caspase-10. And *in vitro* experiments further demonstrated that ectopic expression of miR-421 significantly downregulated the transcription and translation of caspase-10. Given that caspase-10 is one of the upstream initiators of apoptosis [10; 11], it is entirely possible that miR-421 inhibits cell apoptosis through restraining the expression of caspase-10.

In conclusion, our study indicates that miR-421 plays an oncogenic role in breast cancer, probably through repressing caspase-10 related apoptosis signaling. Therefore, targeting miR-421/caspase-10 might be a potential strategy for patients with breast cancer.

References

[1] DONEPUDI MS, KONDAPALLI K, AMOS SJ, VENKANTESHAN P. Breast cancer statistics and markers. J Cancer Res Ther 2014; 10: 506–511. doi: 10.4103/0973-1482.137927

[2] FABIAN MR, SONENBERG N, FILIPOWICZ W. Regulation of mRNA translation and stability by microRNAs. Annu Rev Biochem 2010; 79: 351–379. doi: 10.1146/annurev-biochem-060308-103103

[3] CASCIONE L, GASPARINI P, LOVAT F, CARASI S, PULVIRENTI A et al. Integrated microRNA and mRNA signatures associated with survival in triple negative breast cancer. PLoS one 2013; 8: e55910. doi: 10.1371/journal.pone.0055910

[4] LEREBOURS F, CIZERON-CLAIRAC G, SUSINI A, VACHER S, MOURET-FOURME E et al. miRNA expression profiling of inflammatory breast cancer identifies a 5-miRNA signature predictive of breast tumor aggressiveness. Int J Cancer 2013; 133: 1614–1623. doi: 10.1002/ijc.28171

[5] LU M, KONG X, WANG H, HUANG G, YE C et al. A novel microRNAs expression signature for hepatocellular carcinoma diagnosis and prognosis. Oncotarget 2017; 8: 8775–8784. doi: 10.18632/oncotarget.14452

[6] ZHOU S, WANG B, HU J, ZHOU Y, JIANG M et al. miR-421 is a diagnostic and prognostic marker in patients with osteosarcoma. Tumour Biol 2016; 37: 9001–9007. doi: 10.1007/s13277-015-4578-5

[7] CINEGAGLIA NC, ANDRADE SC, TOKAR T, PINHEIRO M, SEVERINO FE et al. Integrative transcriptome analysis identifies deregulated microRNA-transcription factor networks in lung adenocarcinoma. Oncotarget 2016; 7: 28920–28934. doi: 10.18632/oncotarget.8713

[8] GE X, LIU X, LIN F, LI P, LIU K et al. MicroRNA-421 regulated by HIF-1alpha promotes metastasis, inhibits apoptosis, and induces cisplatin resistance by targeting E-cadherin and caspase-3 in gastric cancer. Oncotarget 2016; 7: 24466–24482. doi: 10.18632/oncotarget.8228

[9] HAO J, ZHANG S, ZHOU Y, LIU C, HU X et al. MicroRNA 421 suppresses DPC4/Smad4 in pancreatic cancer. Biochem Biophys Res Commun 2011; 406: 552–557. doi: 10.1016/j.bbrc.2011.02.086

[10] COHEN GM. Caspases: the executioners of apoptosis. Biochem J 1997; 326: 1–16.

[11] WILSON NS, DIXIT V, ASHKENAZI A. Death receptor signal transducers: nodes of coordination in immune signaling networks. Nat Immunol 2009; 10: 348–355. doi: 10.1038/ni.1714

[12] PAN Y, JIAO G, WANG C, YANG J, YANG W. MicroRNA-421 inhibits breast cancer metastasis by targeting metastasis associated 1. Biomed Pharmacother 2016; 83: 1398–1406. doi: 10.1016/j.biopha.2016.08.058

[13] SHEN XG, WANG C, LI Y, ZHOU B, XU B et al. Downregulation of caspase-10 predicting poor survival after resection of stage II colorectal cancer. Int J Colorectal Dis 2011; 26: 1519–1524. doi: 10.1007/s00384-011-1239-z

[14] BUENO RC, CANEVARI RA, VILLACIS RA, DOMINGUES MA, CALDEIRA JR et al. ATM down-regulation is associated with poor prognosis in sporadic breast carcinomas. Ann Oncol 2014; 25: 69–75. doi: 10.1093/annonc/mdt421

[15] HU H, DU L, NAGABAYASHI G, SEEGER RC, GATTI RA. ATM is down-regulated by N-Myc-regulated microRNA-421. Proc Natl Acad Sci U S A 2010; 107: 1506–1511. doi: 10.1073/pnas.0907763107

[16] LI Y, LI W, ZHANG JG, LI HY, LI YM. Downregulation of tumor suppressor menin by miR-421 promotes proliferation and migration of neuroblastoma. Tumour Biol 2014; 35: 10011–10017. doi: 10.1007/s13277-014-1921-1

[17] CHEN L, TANG Y, WANG J, YAN Z, XU R. miR-421 induces cell proliferation and apoptosis resistance in human nasopharyngeal carcinoma via downregulation of FOXO4. Biochem Biophys Res Commun 2013; 435: 745–750. doi: 10.1016/j.bbrc.2013.05.056

[18] ENGELS IH, TOTZKE G, FISCHER U, SCHULZE-OSTHOFF K, JANICKE RU. Caspase-10 sensitizes breast carcinoma cells to TRAIL-induced but not tumor necrosis factor-induced apoptosis in a caspase-3-dependent manner. Mol Cell Biol 2005; 25: 2808–2818.