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Original Article

Downregulation of miRNA-449a expression associated with advanced stages and lymph node metastasis of breast cancer

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Abstract: Accumulating evidence has revealed that miR-449a expression was downregulated in various human cancers, downregulation of which was associated with cancer development and progression. This study assessed miR-449a expression in different molecular subtypes of breast cancer tissue specimens and explored miR-449a-targeting genes to predict novel gene pathways involved in breast cancer. Paired tissue specimens from 58 breast cancer patients were collected for qRT-PCR analysis of miR-449a expression and gene ontology, KEGG analyses were performed to predict miR-449a-targeting genes and pathways with different web-based tools. The data showed that miR-449a expression was reduced in breast cancer tissue specimens compared to the corresponding adjacent breast tissues ($P < 0.05$) and miR-449a was significantly down-regulated in all four different molecular subtypes of breast cancer, in particular, in triple negative breast cancer. Level of miR-449a was also lower in the breast cancer cell line MDA-MB-231 than that of MCF-7 cell line. Reduced miR-449a expression was significantly associated with advanced breast cancer TNM staging and lymph node metastasis ($P < 0.05$). The gene ontology, KEGG pathway and network analytic data showed that miR-449a could regulate genes that are involved in cell proliferation and transcription regulator activity, sequence-specific DNA binding and enzyme binding, cytoskeleton, endocytosis, Notch signaling pathway, p53 signaling pathway, and MAPK signaling pathway. Hub genes (such as SRC, NOTCH1, BCL2, HDAC1, CCND1, and CDK6) may play critical roles in their co-regulatory networks. Collectively, these data suggest that detection of miR-449a expression could serve as a tumor marker in the prediction of breast cancer progression and metastasis. Further investigation of miR-449a and miR-449a-targeting genes and pathways could provide novel strategies for breast cancer patients.

Keywords: miR-449a, breast cancer, target genes, molecular subtypes, clinicopathological parameters

Introduction

Breast cancer is a significant worldwide health problem and accounts for 1.4 million new cases per year, the highest global incidence rate in women [1, 2]. Breast cancer is the second highest cancer-related mortality amongst women in the world and there are an estimated 458,400 cancer-related deaths globally in 2008 [1, 2]. Advancement in breast cancer early screening and detection, targeted therapy options, and cancer prevention, survival of breast cancer patients has been dramatically improved over the last two decades. Diagnosis of breast cancer at the early stages also significantly increases the rate of curable surgery, however tumor reoccurrence is also a possibility. Thus, identification and evaluation of biomarkers to predict breast cancer progression and responses to treatment could be beneficial to both clinicians and patients. To this end, previous studies have indicated that profiles of microRNA (miRNA) could be useful and valuable in order to identify and evaluate molecular markers for such purposes [3, 4].

miRNAs are a class of eukaryotic endogenous small non-coding RNAs, 18-25 nucleotides in length and functionally target the 3'-untranslational region of targeting mRNA to block mRNA

Table 1. Association of miR-449a expression with molecular subtypes of breast cancer patients

	N	Level of miR-449a expression*	*p* value
Molecular subtypes	58	0.36 ± 0.35	< 0.001
luminal A	12	0.47 ± 0.39	0.002
luminal B	15	0.35 ± 0.43	< 0.001
HER2+	14	0.32 ± 0.34	< 0.001
TNBC	17	0.31 ± 0.25	< 0.001

*Normalized to that of the non-cancerous breast tissues.

translation and promote mRNA degradation [5]. Emerging data indicate that a single miRNA can regulate tens or even hundreds of targeting mRNAs; conversely, a gene can also be targeted by multiple miRNAs [6]. Therefore, miRNAs play a critical role in regulation of diverse biological processes, such as cell proliferation, differentiation, apoptosis, and autophagy [7-9]. Additionally, miRNAs also have the ability to act as oncogenes and tumor suppressors [10, 11]. Dysregulation of miRNA expression has been reported to be associated with the development of a large number of human cancers [12]. In this study, we focused on a particular miRNA, miR-449a, which is localized at chromosome 5q11.2. Previous studies demonstrated that miR-449a expression was downregulated in a variety of solid tumors, including ovarian cancer [13], hepatocellular carcinoma [14], bladder cancer [11], non-small cell lung cancer [15], and colorectal cancer [16]. miR-449a functions as a tumor-suppressor in gastric adenocarcinoma and osteosarcoma via targeting BCL2 expression [17, 18], and other studies have also demonstrated that miR-449a functions as a tumor suppressor or possessed tumor-suppressive activities in a variety of human cancers [13-18]. In breast cancer, Yang X et al. showed that miR-449a/b was able to target and inhibit expression of the oncogenic proteins CDK6 and CDC25A, resulting in cell cycle arrest at the G1 phase in the breast cancer cell line MCF-7 [19]. However, to date, there are no studies assessing miR-449a expression in breast cancer tissue. Thus, in this study, we firstly analyzed miR-449a expression in different invasive breast cancer tissues compared to paired non-cancerous breast tissues specimens for association with clinicopathological data from patients, and then performed bioinformatical analysis to predict miR-449a-targeting genes and related gene pathways.

Material and methods

Patients and tumor specimens

Fifty-eight paired breast cancer and adjacent non-cancerous breast tissues from female breast cancer patients were obtained from Department of Pathology, The First Affiliated Hospital of Guangxi Medical University between April 2013 and March 2015. The median age of these patients was 48 years old (range 24 to 81 years old) and all patients were diagnosed with invasive breast ductal carcinoma according to the 7th edition of the American Joint Committee on Cancer (AJCC) staging manual [20, 21]. On examination of immunohistochemical data on estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2) expression in pathology reports, 12 (20.7%) were diagnosed with Luminal A, 15 (25.9%) were Luminal B, 14 (24.1%) were HER2 overexpression (HER2+) and 17 (29.3%) were triple negative breast cancer (TNBC). All samples were collected before any radiation therapy, chemotherapy or endocrine therapy. This study was reviewed and approved by The Ethics Committee of the First Affiliated Hospital of Guangxi Medical University (Nanning, Guangxi, China). All patients provided informed consent before participation in this study.

Cell lines and culture

Human breast cancer cell line MDA-MB-231 and MCF-7 were obtained from Shanghai Fudan Breast Cancer Institute (Shanghai, China) and cultured in Dulbecco's Modified Eagle's Medium (DMEM; Invitrogen, Carlsbad, CA, USA) supplemented with 15% fetal bovine serum (FBS; Invitrogen) and 100 U/ml penicillin (Invitrogen), and 100 μg/ml streptomycin (Invitrogen) in a humidified incubator with 5% CO_2 at 37°C.

RNA isolation and quantitative RT-PCR

Total RNA was isolated and small RNA was enriched from 58 paired FFPE breast cancer samples and corresponding adjacent breast tissues by using the miRNeasy FFPE Kit (Cat# 217504; Qiagen, Hilden, Germany). RNA was isolated from cell lines with the RNeasy Mini Kit

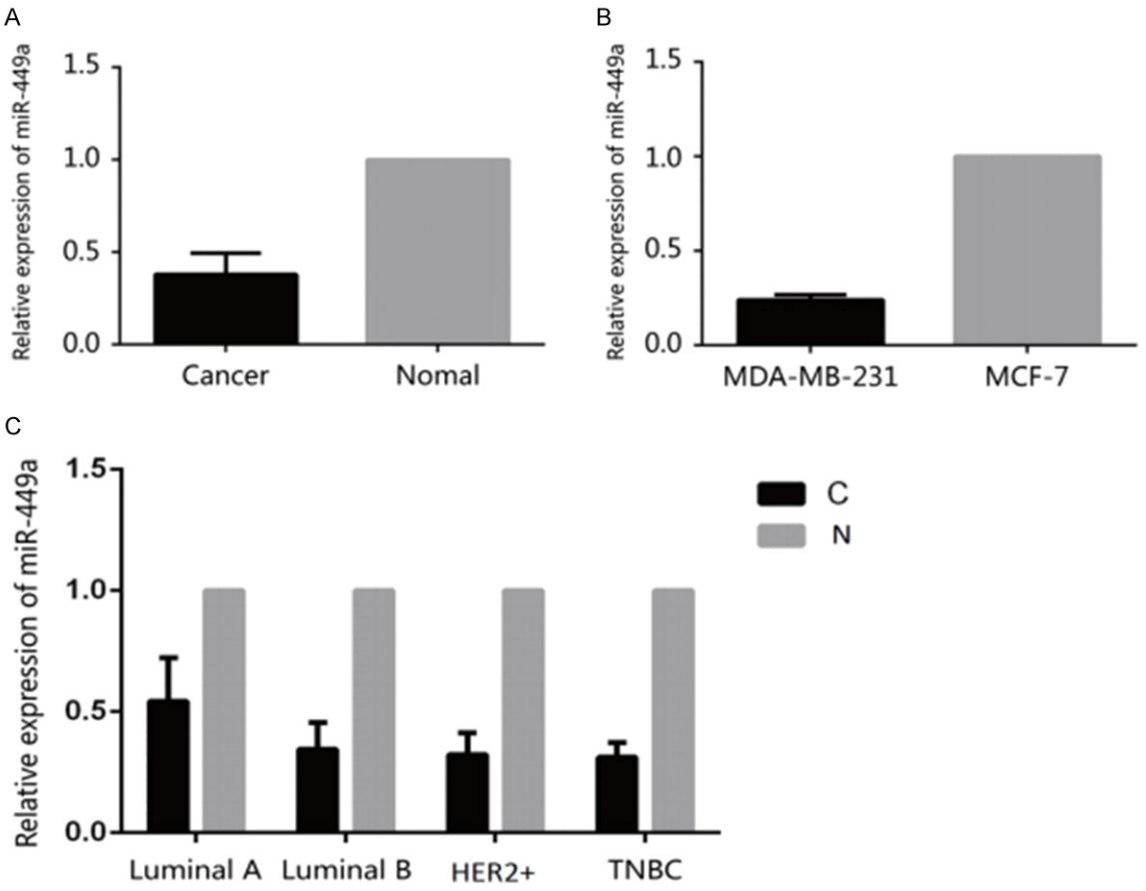

Figure 1. Downregulation of miR-449a expression in human breast cancer tissues and cell lines. A. Relative levels of miR-449a expression were assessed by using qRT-PCR in 61 paired breast cancer and non-cancerous tissue specimens. B. Level of miR-449a expression was assessed in two breast cancer cell lines (MDA-MB-231 and MCF-7) using qRT-PCR. C. Comparison of miR-449a expression in different molecular subtypes of breast cancer compared to non-cancerous breast tissues. Expression of miR-449a was normalized to an endogenous control RNU6B and data represent the $2^{-\Delta\Delta Ct}$ values with mean ± SD.

(Cat# XYGEN Ambion, Austin, TX, USA) following to the manufacturer's instructions. RNA samples were quantified with a spectrometer (Thermo, Waltham, MA, USA) and then reversely transcribed to cDNA by using the miScript Reverse Transcription Kit (Cat# 218161; Qiagen) according to the manufacturer's protocol.

To assess miR-449a level, we performed qPCR using the miScript SYBR Green PCR Kit (Cat# 218073; Qiagen) in ABI StepOne Real-time PCR system (Applied Biosystems, Foster city, CA, USA). Specific primers Hs_miR-449_1 and endogenous control Hs_RNU6-2_11 were also obtained from Qiagen (Cat# MS00004228 and 218193, respectively). qPCR conditions were set to an initial 95°C for 15 min, followed by 40 cycles of 94°C for 15 s, 55°C for 30 s, and 70°C for 30 s. The relative quantification of miR-449a level was calculated using $2^{-\Delta\Delta Ct}$ cycle threshold method. A fold change expression of less than 1 was noted as low expression for miR-449a.

Gene ontology (GO) and KEGG pathway and network analysis

To predict miR-449a-targeting genes and pathways, we performed searches using TargetScan (http://www.targetscan.org/), miRanda (http://www.microrna.org/microrna/home.do), miRBase (http://www.mirbase.org/), starBase (http://starbase.sysu.edu.cn/), and miRDB (http://mirdb.org/miRDB/), Tarbase (http://microrna.gr/tarbase/), mirTarBase (http://mirtarbase.mbc.nctu.edu.tw/index.html). All miR-449a targeting genes were identified and path-

Table 2. Association of miR-449a expression with clinicopathological data from patients

Characteristics	N	Level of miR-449a expression		*p* value
		low, n (%)	high, n (%)	
Age (years)				
≤ 48	30	14 (48.3)	16 (55.2)	0.79
> 48	28	15 (51.7)	13 (44.8)	
Tumor size (cm)				
≤ 5	41	18 (62.1)	23 (79.3)	0.25
> 5	18	11 (37.9)	6 (20.7)	
Histological grade				
I	5	3 (10.3)	2 (6.9)	1
II+III	56	28 (89.7)	28 (93.1)	
TNM staging				
I+II	48	20 (69)	28 (96.6)	0.012
III+IV	10	9 (31)	1 (3.4)	
Lymph node metastasis				
Yes	21	20 (69)	11 (38)	0.034
No	37	10 (31)	18 (62)	
ER status				
Positive	24	11 (37.9)	13 (44.8)	0.79
Negative	34	18 (62.1)	16 (55.2)	
PR status				
Positive	25	12 (41.4)	13 (44.8)	1.00
Negative	33	17 (58.6)	16 (55.2)	
HER2 status				
Positive	24	13 (44.8)	11 (37.9)	0.79
Negative	34	16 (55.2)	18 (62.1)	

way and network analyses were subsequently performed by GO and KEGG with DAVID (https://david.ncifcrf.gov/).

Statistical analysis

All statistical analyses were performed with SPSS version 21.0 (SPSS Inc., Chicago, IL, USA). Data were presented as the mean ± SD or the median for numeric parameters. Each experiment was repeated at least three times. The paired samples *t* test was used to compare levels of miR-449a expression between breast cancer and the corresponding adjacent normal tissues. One-way ANOVA was employed to analyze differences of miR-449a expression between different molecular subtypes of breast cancer tissues. The Pearson (χ^2) chi-square or Fisher exact test was performed to analyze the association of miR-449a expression with clinicopathological data. A statistical significance was defined as $P < 0.05$.

Results

Downregulation of miR-449a expression in different molecular subtypes of breast cancer tissues and cell lines

In this study, we assessed the level of miR-449a expression in breast cancer compared to non-cancerous breast tissue specimens using qRT-PCR. Our data showed that the level of miR-449a was significantly down-regulated in breast cancer tissues compared to the corresponding adjacent breast tissues ($P < 0.05$; **Table 1** and **Figure 1A**). Expression of miR-449a was lower in the breast cancer cell line MDA-MB-231 (an ER-, PR-, and HER2- cell line) than that in MCF-7 cell line (an ER+, PR+, and HER2+ cell line) (**Figure 1B**). Furthermore, level of miR-449a was lower in luminal A ($P = 0.002$), luminal B ($P < 0.001$), HER2 overexpressing ($P < 0.001$), and triple-negative breast cancer ($P < 0.001$) tissues (**Table 1** and **Figure 1C**). The One-way ANOVA data showed that there was no difference in miR-449a expression among these four subtypes of breast cancer tissue specimens ($P > 0.05$).

Association of miR-449a levels with clinicopathological data from patients

We next associated miR-449a levels with clinicopathological data from patients using a cut-off point of the median value of relative miR-449a level of 0.236 ($2^{-\Delta\Delta Ct}$) as low and high miR-449a expression. Our data showed that the mean levels of low and high miR-449a expression were 0.108 and 0.682, respectively (**Table 2**). The low level of miR-449a expression was associated with advanced breast cancer tumor node metastasis (TNM) stages ($P = 0.012$) and lymph node metastasis ($P = 0.034$). However, the level of miR-449a expression was not associated with other parameters such as age, tumor size, histological grade, pathologic types and ER status, PR status, HER2 status (**Table 2**).

Prediction of miR-449a-targeting genes and pathways

miRNA functions to post-transcriptionally regulate expression of protein-coding genes [22]. We thus, performed a bioinformatics analysis to predict miR-449a-targeting genes and path-

Table 3. GO analysis of the dysregulated miR-449a-targeting genes and their functions

GO ID	GO biological process	GO ontology	Gene number (%)	*p* value	Related genes
GO:0042127	Regulation of cell proliferation	Biological process	38 (10.3)	4.70E-06	E2F3, MARCKSL1, KITLG etc.
GO:0006357	Regulation of transcription from RNA polymerase II promoter	Biological process	31 (8.4)	3.94E-04	HOXA13, ONECUT2, MED22 etc.
GO:0010605	Negative regulation of macromolecule metabolic process	Biological process	29 (7.9)	0.00199338	CBX3, ITGB3, TNRC6B etc.
GO:0010604	Positive regulation of macromolecule metabolic process	Biological process	32 (8.7)	0.002680006	E2F3, HOXA13, ONECUT2 etc.
GO:0010557	Positive regulation of macromolecule biosynthetic process	Biological process	26 (7)	0.003418249	E2F3, HOXA13, ONECUT2 etc.
GO:0046907	Intracellular transport	Biological process	26 (7)	0.003605409	PACS1, ABCD1, COPZ1 etc.
GO:0007049	Cell cycle	Biological process	27 (7.3)	0.01463802	TXNIP, E2F2, E2F3 etc.
GO:0006355	Regulation of transcription, DNA-dependent DNA	Biological process	51 (13.8)	0.021939327	E2F2, POU6F1, E2F3 etc.
GO:0006796	Phosphate metabolic process	Biological process	31 (8.4)	0.025984583	PPP2R3A, STK35, MKNK2 etc.
GO:0006793	Phosphorus metabolic process	Biological process	31 (8.4)	0.025984583	PPP2R3A, STK35, MKNK2 etc.
GO:0051252	Regulation of RNA metabolic process	Biological process	51 (13.8)	0.031488355	E2F2, POU6F1, E2F3 etc.
GO:0016310	Phosphorylation	Biological process	26 (7)	0.034756222	STK35, MKNK2, TTN etc.
GO:0045449	Regulation of transcription	Biological process	69 (18.7)	0.035798059	E2F2, POU6F1, E2F3 etc.
GO:0008104	Protein localization	Biological process	28 (7.6)	0.036249861	PACS1, CHMP7, COPZ1 etc.
GO:0019899	Enzyme binding	Molecular function	23 (6.2)	0.001027215	PTPRK, MAP2K1, STRN3 etc.
GO:0043565	Sequence-specific DNA binding	Molecular function	24 (6.5)	0.003107002	ZNF281, POU6F1, SATB2 etc.
GO:0003700	Transcription factor activity	Molecular function	33 (8.9)	0.004841857	E2F2, POU6F1, E2F3 etc.
GO:0030528	Transcription regulator activity	Molecular function	43 (11.7)	0.021850431	E2F2, POU6F1, CAMTA1 etc.
GO:0004672	Protein kinase activity	Molecular function	21 (5.7)	0.022891328	PRKCA, PAN3, MAP2K1 etc.
GO:0042802	Identical protein binding	Molecular function	21 (5.7)	0.036690598	ALDOA, SHMT1, SYT1 etc.
GO:0005856	Cytoskeleton	Cellular component	44 (11.9)	0.004195917	ALDOA, LIMA1, KITLG etc.
GO:0005794	Golgi apparatus	Cellular component	28 (7.6)	0.023743329	PACS1, MGAT5B, GALNT7 etc.
GO:0005654	Nucleoplasm	Cellular component	28 (7.6)	0.027179242	E2F2, E2F3, E2F5, MED22 etc.
GO:0000267	Cell fraction	Cellular component	32 (8.7)	0.041046346	SLC6A1, SGPP1, MED22 etc.

Table 4. KEGG pathway analysis of miR-449a targeting genes

GO ID	KEGG_pathway	Gene number (%)	*p* value	Genes
hsa05220	Chronic myeloid leukemia	11 (2.9)	9.00E-06	E2F2, E2F3, CCND1, CDKN1B, MAP2K1, HDAC1, TGFBR2, SMAD4, CDK6, CDK4, MYC
hsa04110	Cell cycle	13 (3.5)	3.46E-05	CCNE2, E2F2, E2F3, CCND1, YWHAG, CDKN1B, HDAC1, E2F5, SMAD4, CDK6, CDK4, MYC, CDC25A
hsa05222	Small cell lung cancer	10 (2.7)	1.49E-04	COL4A4, CCNE2, E2F2, E2F3, CCND1, CDKN1B, BCL2, CDK6, CDK4, MYC
hsa05200	Pathways in cancer	20 (5.4)	2.30E-04	PRKCA, COL4A4, E2F2, E2F3, MAP2K1, MET, TGFBR2, SMAD4, KITLG, LEF1, CDK6, CDK4, CCNE2, CCND1, CDKN1B, HDAC1, BCL2, PDGFRA, PTCH1, MYC
hsa05215	Prostate cancer	10 (2.7)	2.33E-04	CCNE2, E2F2, E2F3, CCND1, CDKN1B, MAP2K1, BCL2, PDGFRA, LEF1, CREB5
hsa05214	Glioma	8 (2.2)	6.66E-04	PRKCA, E2F2, E2F3, CCND1, MAP2K1, PDGFRA, CDK6, CDK4
hsa05210	Colorectal cancer	9 (2.4.)	7.84E-04	CCND1, MAP2K1, BCL2, MET, TGFBR2, PDGFRA, SMAD4, LEF1, MYC
hsa05218	Melanoma	8 (2.2)	0.001367481	E2F2, E2F3, CCND1, MAP2K1, MET, PDGFRA, CDK6, CDK4
hsa05212	Pancreatic cancer	8 (2.2)	0.001485354	E2F2, E2F3, CCND1, MAP2K1, TGFBR2, SMAD4, CDK6, CDK4
hsa05223	Non-small cell lung cancer	7 (1.9)	0.001628239	PRKCA, E2F2, E2F3, CCND1, MAP2K1, CDK6, CDK4
hsa05219	Bladder cancer	6 (1.6)	0.002953672	E2F2, E2F3, CCND1, MAP2K1, CDK4, MYC
hsa04144	Endocytosis	12 (3.3)	0.004102277	DNM3, IL2RB, DNM1L, MET, TGFBR2, PDGFRA, RAB11B, VPS4A, VPS37B, VPS37D, HSPA1B, SRC
hsa04330	Notch signaling pathway	6 (1.6)	0.004846299	NCSTN, NOTCH1, HDAC1, DLL1, JAG1, NUMBL
hsa04115	p53 signaling pathway	7 (1.9)	0.005267877	CCNE2, CCND1, SERPINE1, CDK6, MDM4, CDK4, SESN2
hsa04510	Focal adhesion	11 (2.9)	0.020691955	PRKCA, COL4A4, CCND1, VAV3, MAP2K1, BCL2, MET, PDGFRA, ITGA11, ITGB3, SRC
hsa04010	MAPK signaling pathway	13 (3.5)	0.024150299	PRKCA, RPS6KA4, MAP2K1, MAPT, TGFBR2, PDGFRA, MKNK2, RRAS, CACNA1E, HSPA1B, PLA2G2D, MYC, PLA2G2F
hsa05216	Thyroid cancer	4 (1.1)	0.030617522	CCND1, MAP2K1, LEF1, MYC
hsa04520	Adhesion junction	6 (1.6)	0.035617765	PVRL1, MET, TGFBR2, SMAD4, LEF1, SRC
hsa04710	Circadian rhythm	3 (0.8)	0.036907881	CRY2, PER2, CLOCK

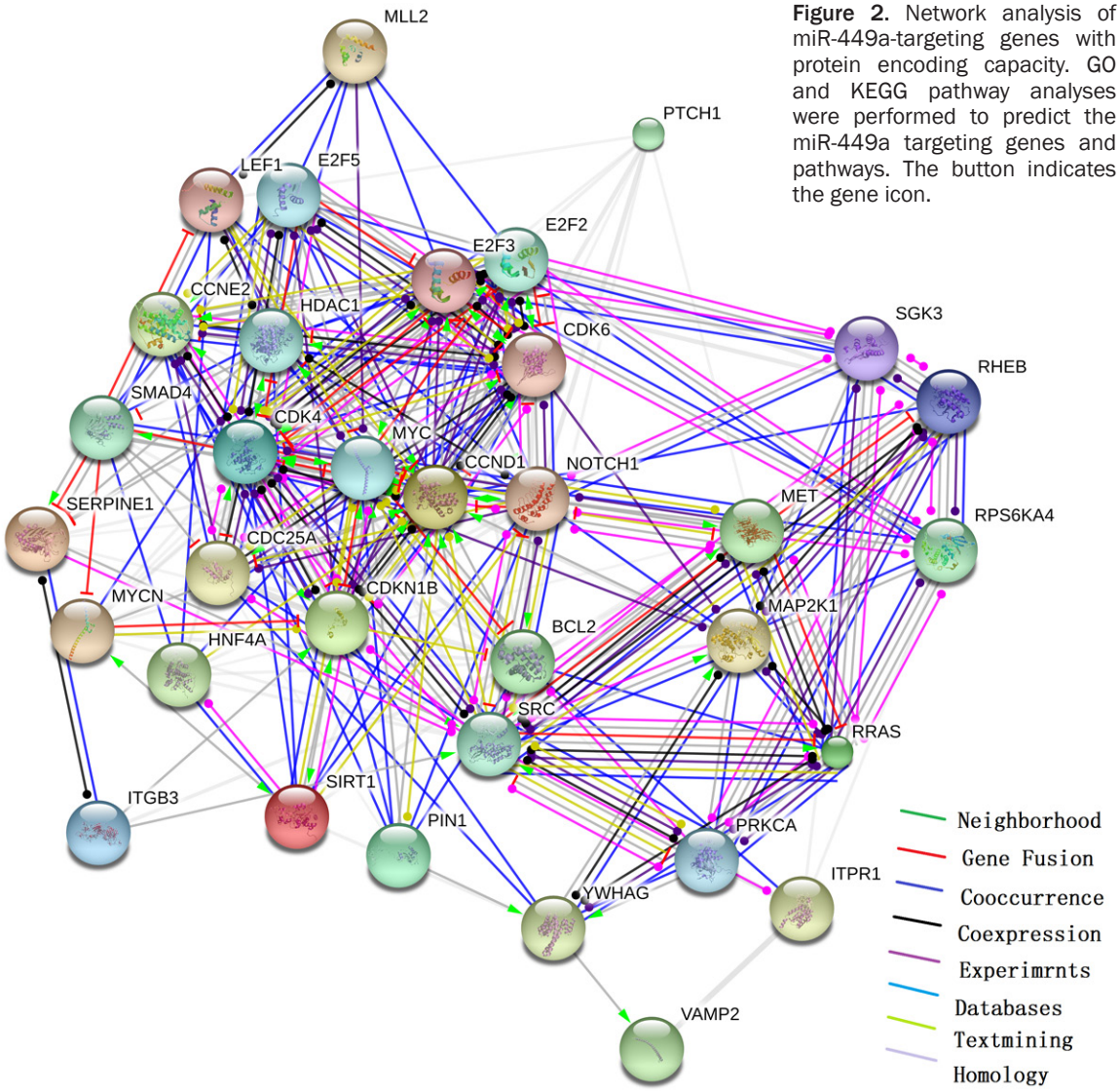

Figure 2. Network analysis of miR-449a-targeting genes with protein encoding capacity. GO and KEGG pathway analyses were performed to predict the miR-449a targeting genes and pathways. The button indicates the gene icon.

ways using the online tools TargetScan, miRanda, miRBase, starBase and miRDB. We assessed these five computational algorithms and obtained 4363, 7010, 844, 2072, and 535 genes, respectively. After intersection, there were 67 genes potentially identified as miR-449a-targeting genes. After these genes were overlapped with validated targeting genes from other two databases (Tarbase with 218 genes and mirTarBase of 159 genes), we obtained a total of 413 potential miR-449a-targeting genes for the subsequent GO, KEGG pathway and network analyses.

The GO analysis categorized these genes to functions in biological process (BP), cellular component (CC) and molecular function (MF) ($P < 0.05$; **Table 3**) and showed that miR-449a-targeting genes were involved in cell proliferation and macromolecule biosynthetic and metabolic processes. Cellular functions were predominantly associated with Golgi apparatus and the cytoskeleton; and molecular functions included transcription activity and sequence-specific DNA binding (**Table 3**). Subsequent KEGG pathway analysis indicated that there were 23 pathways available, out of which 19 signaling pathways were statistically significant ($P < 0.05$; **Table 4**). The results indicated that miR-449a may be involved in several signaling pathways, such as the cell cycle, focal adhesion, endocytosis, p53 signaling pathway, the MAPK signaling pathway, the Notch signaling pathway and pathways involved in cancer, such as chronic myeloid leukemia and prostate cancer. Additionally, we also identified 35 hub genes by final network analysis and found that SRC, NOTCH1, BCL2, HDAC1, CCND1, CDK6 were the top six

interaction gene counts among the overlapping genes that play important roles in the stability of gene regulatory networks (**Figure 2**).

Discussion

Breast cancer is a highly heterogeneous disease and according to the status of ER, PR, and HER2 expression, breast cancer can be classified into four different subtypes, i.e., luminal A (ER+/PR+/HER2-), luminal B (ER+/PR+/HER2+), HER2 overexpression (ER-/PR-/HER2+), and triple negative breast cancer (TNBC) (ER-/PR-/HER2-) [23, 24]. Clinically, breast cancer has different treatment options for these molecular subtypes. Generally, receptor-positive breast cancers (ER+/PR+/HER2+) have better overall and disease-free survival rates after surgery, along with targeted molecular therapies [25, 26]. For example, a monoclonal anti-HER2/neu antibody, trastuzumab, can target effectively HER2-positive breast cancer and prolong survival in both early and advanced stages of disease [25, 27]. Moreover, patients with ER+/HER2+ breast cancers also benefit better from drugs that inhibit the PI3K/AKT molecular pathway than that of ER-/HER2+ breast cancer [28]. Thus, clinically, prevention and control of ER-, PR-, and/or HER2- breast cancer are a challenge to medical oncologists. In our current study, we assessed miR-449a expression in these subtypes of invasive breast cancer tissue specimens compared to non-cancerous breast tissue in order to identify a biomarker to predict progression of these different subtypes of breast cancer. Our data showed that miR-449a level was significantly reduced in all four different molecular subtypes of breast cancer tissue specimens compared to the corresponding adjacent non-cancerous breast tissues. Expression of miR-449a was also lower in MDA-MB-231 cells compared to the MCF-7 cell line. Downregulation of miR-449a expression was significantly associated with advanced TNM stages and lymph node metastasis of breast cancer. Furthermore, our GO and KEGG pathway and network analysis showed that miR-449a could regulate genes that are involved in cell proliferation and transcription regulator activity, sequence-specific DNA binding and enzyme binding, cytoskeleton, endocytosis, Notch signaling pathway, p53 signaling and MAPK signaling pathways. The hub genes (SRC, NOTCH1, BCL2, HDAC1, CCND1, and CDK6) may play critical roles in their co-regulatory networks. In conclusion, these data suggest that miR-449a expression could serve as a tumor marker for breast cancer progression. However, further investigation of these genes and pathways is needed to disclose whether targeting of these genes and pathways could provide novel strategies for breast cancer patients.

In a clinical and experimental setting, there has been a rapid development of modern bioinformatics and the advancement in high-throughput technologies, such as gene chips, to analyze expression of altered genes for subtyping, and differentially diagnosing breast cancers [29, 30]. Such molecular analyses may help oncologists to select treatment and predict treatment responses or cancer progression and prognosis. For example, several decades ago, breast cancer was subtyped into ER-, PR-, and/or HER2- according to the expression of these receptors in breast cancer cells, which revolutionized molecular therapy of breast cancer and dramatically improved survival of patients with ER+, PR+, and/or HER2+ breast cancer [31]. To date, it has been established that different molecular subtypes of breast cancer will have different prognoses and therapeutic options, although other therapy, such as surgery, or other treatments, such as endocrine and chemoradiation therapies also significantly reduced breast cancer mortality rate [32]. Recently, a large number of studies have reported dysregulated expression of miRNAs and the potential target genes in different human cancers, including breast cancer [33-38]. In the current study, we focused on altered expression of miR-449a in breast cancer and found that miR-449a level was significantly reduced in all four different molecular subtypes of breast cancer tissue specimens compared to the corresponding adjacent breast tissues. Downregulation of miR-449a expression was significantly associated with advanced TNM stages and lymph node metastasis of breast cancer. Our current data are in agreement with previous studies in ovarian [39], prostate [40], and endometrial cancers [36]. Further studies will investigate the underlying mechanisms responsible for reduced miR-449a expression in breast cancers.

Our current study analyzed miR-449a-targeting genes using seven different computational

algorithms. Overlapping genes were then analyzed systematically using a series of data-filtering methods, including GO and KEGG pathway and DAVID network analyses. We predicted that functions of these miR-449a targeting genes were associated with cell proliferation, transcription regulator activity and cytoskeleton. Pathway analysis indicated that miR-449a may be involved in several signaling pathways, such as the p53 signaling pathway, cell cycle, focal adhesion, the MAPK signaling pathway, and the Notch signaling pathway. Indeed, previous studies demonstrated that the p53 signaling pathway, the MAPK signaling pathway, and/or the Notch signaling pathway were associated with breast cancer development and progression [41-43]. Moreover, our current data revealed that SRC, NOTCH1, BCL2, HDAC1, CCND1, and CDK6 genes were the key genes among the all of 413 miR-449a-targeting genes and play a vital role in stabilizing structures in the interaction gene network. This finding is consistent with the data reported previously by others [18, 19, 39, 40]. Furthermore, previous studies have demonstrated that mR-449a is considered to be a tumor-suppressor gene and could target expression of Myc-associated zinc-finger protein (MAZ), participating in the PI3K/AKT pathway in glioblastomas [44], while miR-449a expressions was elevated after p53 activation and their inactivation may contribute to carcinogenesis and progression of serous ovarian carcinoma [13]. miR-449a was also reported to be involved in various biological processes such as cell mobility and proliferation [45, 46]. Chen SP et al. have demonstrated that down-regulation of miR-449a in hepatocellular carcinoma cells was associated with tumor cells undergoing epithelial-mesenchymal transition [14], while miR-449a gene GG genotype increased the risk of developing gastric cancer in a Chinese population [47]. Taken together, miR-449a could be a candidate tumor suppressor miRNA in breast cancer. However, additional experiments are needed to confirm these bioinformatics-generated data.

However, our current study does have some limitations that should be considered in the interpretation of our findings. For example, there was no significantly statistical difference in miR-449a level among these four molecular subtypes of breast cancer, which may be due to the small sample size and lack of analysis of other types of breast cancer. Thus, further studies are warranted to rule out the contribution of confounding factors before drawing a clear conclusion. Furthermore, the case distribution of breast cancer subtypes was not consistent with those observed in other studies, e.g., Li J, et al. showed that frequency of luminal A, luminal B, HER2-overexpressed, triple negative, and non-luminal HER2-positive subtypes were: 35.6%, 22.5%, 13.1%, 15.2%, and 13.7%, respectively [48]. Although dysregulation of miR-449a has been observed in breast cancer, there is little knowledge regarding the molecular mechanisms and specific signaling pathways by which miR-449a modulates breast cancer progression.

In conclusion, our current data suggest that detection of miR-449a expression may be a useful biomarker in prediction of breast cancer progression and metastasis. Further investigation of these miR-449a-targeting genes and pathways could provide novel strategies for treating breast cancer patients.

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Disclosure of conflict of interest

None.

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