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Original Article

Decreased expression of lncRNA MEG3 in breast cancer is associated with poor prognosis

Weifeng Shi[1,2], Suhua Xia[3], Yuan Yin[2], Xiaowei Qi[2], Chungen Xing[1]

[1]Department of General Surgery, The Second Affiliated Hospital of Soochow University, Suzhou, Jiangsu, P. R. China; [2]Department of General Surgery and Pathology, The Affiliated Hospital of Jiangnan University, Wuxi, Jiangsu, P. R. China; [3]Department of Oncology, The First Affiliated Hospital of Soochow University, Suzhou, Jiangsu, P. R. China

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Abstract: Background: Dysregulated long non-coding RNAs (lncRNAs) play critical roles in tumorigenesis and tumor progression. The purpose of this study was to investigate the relationship between lncRNA MEG3 expression and breast cancer (BC) clinicopathological characteristics and prognosis. Methods: Expression levels of lncRNA MEG3 in 257 BC specimens were determined by quantitative real-time PCR (qRT-PCR), to clarify the clinical significance of lncRNA MEG3 in BC, we further explored the relationship between lncRNA MEG3 expression and overall survival (OS) and relapse-free survival (RFS). Results: In the present study, we found that lncRNA MEG3 was down-regulated in BC tissues compared to the adjacent non-tumor tissues. In addition, the decreased lncRNA MEG3 expression was significantly associated with the lymph node metastasis, TNM stage and molecular subtypes ($P < 0.05$). Furthermore, patients with decreased expression of lncRNA MEG3 had poor OS (HR = 3.162, 95% CI = 1.026-9.741, $P = 0.045$), and RFS rates (HR = 2.730, 95% CI = 1.147-6.497, $P = 0.023$). Multivariate Cox proportional hazard model analysis demonstrated that high lncRNA MEG3 expression was an independent poor prognostic factor for BC patients. Conclusions: Our study suggests that decreased expression of lncRNA MEG3 is related to the prognosis of BC; it may be a new prognostic biomarker and potential therapeutic target for BC intervention.

Keywords: Breast cancer, lncRNA MEG3, overall survival, relapse-free survival

Introduction

Breast cancer (BC) is the most common cancer among women and it is estimated there is 231,840 new cases expected to diagnose in 2015 in the USA [1]. Due to improvements in early detection and treatment, the mortality rates of BC have decreased in recent years [1]. However, it is still the second leading cause of cancer death among women. In China, the health burden and morbidity of BC has been increasing during the past 30 years [2]. Because of the population's rising socioeconomic status and unique reproductive patterns, the new diagnosed BCs in China account for 12.2% of all newly diagnosed, and the deaths in China contributes to 9.6% of all deaths from breast cancer worldwide [2]. Therefore, to understand the molecular mechanisms about BC progression will support new useful prognostic biomarker and therapeutic target for BC therapy.

Long non-coding RNAs (LncRNA) are RNA molecules that are longer than 200 nucleotides and are not translated into proteins [3]. Previously, these long non-coding transcripts were considered as simply transcriptional "noise" or cloning artifacts [3]. However, it is realized that lncRNAs play important roles in diverse biological processes during development and disease, such as carcinogenesis [3, 4]. And, many studies have found a myriad of lncRNAs dysregulated in cancers [3, 4]. For example, Ren et al detected lncRNA RNA HOTTIP in colorectal cancer by qRT-PCR, and found lncRNA HOTTIP upregulated in colorectal cancer [5]. Further analysis shown lncRNA HOTTIP overexpression associated with more advanced T stage, clinical stage and distant

Table 1. Correlation between MEG3 expression and clinicopathological characteristics of BC

Characteristics	Meg3 expression		χ^2	*P*
	High expression (89)	Low expression (168)		
Age				
≤ 48 years (130)	49	81	1.090	0.297
> 48 years (127)	40	87		
Histological classification				
I (35)	13	22	0.130	0.937
II (183)	63	120		
III (39)	13	26		
Tumor size				
≤ 2 cm (77)	28	49	0.296	0.862
2-5 cm (160)	55	105		
> 5 cm (20)	6	14		
Lymph nodes status				
Negative (122)	50	72	4.141	0.042
Positive (135)	39	96		
Stage				
I/II (187)	77	110	12.996	0.000
III (70)	12	58		
Menopausal status				
Postmenopausal (102)	30	72	2.035	0.154
Premenopausal (155)	59	96		
Family history				
Yes (50)	18	32	0.051	0.821
No (207)	72	136		
Molecular subtype				
Luminal A (151)	66	85	15.167	0.004
Luminal B (21)	7	14		
Her2 + (37)	8	29		
Basal-like (32)	5	27		
Unclassified (16)	3	13		

metastasis, and predicted poorer prognosis [5]. Chen et al revealed lncRNA CCAT2 was upregulated in cervical squamous cell cancer tissues [6]. And the high lncRNA CCAT2 expression was significantly associated with the FIGO stage, lymph node metastasis and depth of cervical invasion, and predicted poorer prognosis [6]. MEG3 is an imprinted lncRNA expressed from the maternal allele and down regulated in many cancers by promoter methylation, such as bladder cancer, acute myeloid leukemia, pancreatic neuroendocrine tumors, ovarian cancer and hepatocellular carcinoma [7-11]. Studies of re-expression MEG3 in cancer cells show MEG3 is a tumor suppressor gene in these cancers [12, 13]. Unfortunately, the expression and functional role of lncRNAs MEG3 in BC remains largely unknown.

In this study, qRT-PCR assay was performed to detect the expression of lncRNA MEG3 in BC and adjacent non-tumor tissues. Moreover, the correlations of MEG3 expression with clinicopathologic features of BC patients were statistically analyzed. Finally, we determined the potential role of MEG3 in BC prognostic prediction. Our data showed that lncRNA MEG3 was significantly downregulated in BC samples and could be served as a potential molecular biomarker for the prediction of poor prognosis.

Materials and methods

Patients and surgical specimens

257 pairs of BC tissue and matched adjacent normal tissue specimens were collected from patients who underwent surgery between 2008 and 2009 in the Affiliated Hospital of Jiangnan University. The fresh tissue specimens were collected, immediately dropped into liquid nitrogen and conserved in -80°C using refrigerator until use. The clinicopathological variables of patients were shown in **Table 1**. None of the patients recruited in this study had undergone neoadjuvant chemotherapy or radiotherapy. This study was approved by the Research Ethics Committee of the Affiliated Hospital of Jiangnan University. Written informed consent was obtained from all patients. All specimens were handled and made anonymous according to the ethical and legal standards.

RNA isolation and cDNA synthesis

Total RNAs were extracted from tumor tissues and adjacent normal tissues using RNA iso plus reagent (Takara) according to the manufacturer's instructions. The quantity and quality of total RNA was evaluated by measuring the

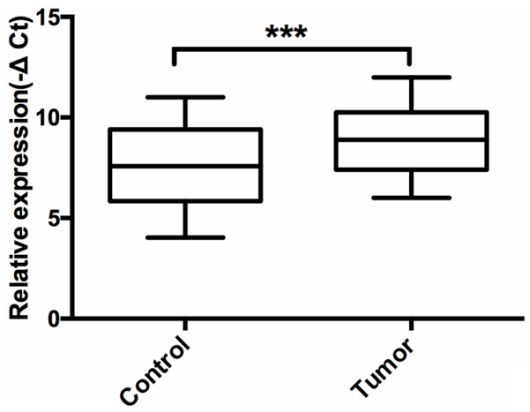

Figure 1. Expression of lncRNA MEG3 is decreased in breast cancer tissues compared with non-malignant tissues through qRT-PCR.

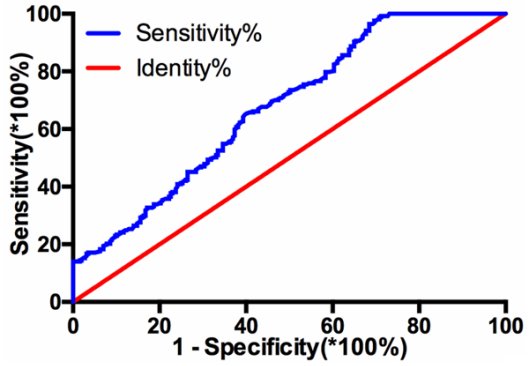

Figure 2. ROC curve analysis of the optimal ΔCt cutoff for predicting survival in patients with breast cancer.

absorbance at 260 and 280 nm. Only samples with an A260:A280 ratio between 1.8 and 2.1 was considered for further analysis. cDNAs were synthesized using the PrimeScript™ RT reagent Kit (Perfect Real Time) (Takara) according to the manufacturer's protocol. Briefly, 1 μg total RNA, 2 μl 5× PrimeScript Buffer (for Real Time), 0.5 μl PrimeScript RT Enzyme Mix I, Oligo dT Primer (50 μM), Random 6 mers (100 μM) and RNase Free dH_2O, were combined in a total reaction volume of 10 μl incubated at 37°C for 15 min, followed by 85°C for 5 s to generate the cDNA.

Quantitative real-time PCR

The expression of lncRNA MEG3 was quantified using SYBR® Fast qPCR Mix (Takara) according to the manufacturer's instructions on the ABI 7500 Real-Time PCR System (Applied Biosystems). Briefly, the 20 μl reaction mixtures were incubated at 95°C for 30 s for the initial denaturation stage, followed by 40 cycles at 95°C for 5 s and 60°C for 34 s. Small nucleolar RNA U6 was used to normalize the target gene expression. The ΔCt method was used to calculate the relative expression of lncRNA MEG3 in BC tissues in comparison with paired normal tissues, respectively. Each sample was examined in triplicate. The primers used in this study were synthesized by Invitrogen with the sequences as follows: 5-'CTGCCCATCTACACCTCACG-3' (forward) and 5'-CTCTCCGCCGTCTGCGCTAGGGGCT-3' (reverse) for MEG3; 5'-GCGCGTCGTGAAGCGT-TC-3' (Forward) and 5'-GTGCAGGGTCCGAGGT-3' (Reverse) for U6.

Statistical analysis

Statistical analysis was conducted using the IBM SPSS Statistics packages 21.0 for mac. Receiver operating characteristics (ROC) analysis with calculation of the Youden index was used to determine the optimal ΔCt cutoff value for estimating high and low expression of MEG3 in BC. The correlation between lncRNA MEG3 expression and clinicopathological features were evaluated by the chi-square test. Overall survival and relapse-free survival were calculated by Kaplan-Meier survival analysis, compared by the log rank test and Cox regression method. All data were presented as mean ± SD. The *P* values less than 0.05 was considered statistically significant.

Results

lncRNA MEG3 was significantly down-regulated in BC tissues

We analyzed the expression levels of lncRNA MEG3 in 257 pairs of BC tissues and adjacent normal tissues from BC patients. As revealed by qRT-PCR analysis, lncRNA MEG3 expression level was significantly lower in BC tissues compared with adjacent normal tissues (**Figure 1**, $P < 0.001$).

ROC analysis of the optimal ΔCt cutoff value for estimating high and low expression of MEG3 in BC

ROC analysis (**Figure 2**) and calculation of the Youden index revealed that the optimal ΔCt cutoff value for estimating high and low expression of MEG3 in BC was 8.065 (area under the curve, 0.675; $P < 0.001$). The calculated sensi-

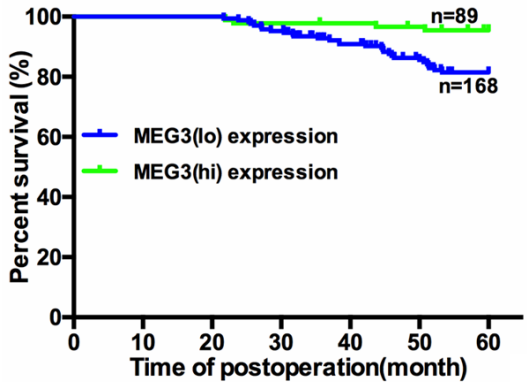

Figure 3. Decreased lncRNA MEG3 expression predicts a poorer overall survival in breast cancer patients.

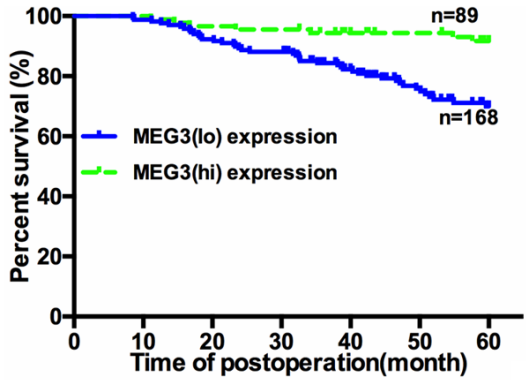

Figure 4. Decreased lncRNA MEG3 expression predicts a poorer relapse free survival in breast cancer patients.

tivity and specificity values for this cutoff were 65.4 and 60.1 %, respectively. BC patients who expressed MEG3 at levels less than the cutoff value were assigned to the low expression group (n = 168), and those with expression above than the cutoff value were assigned to the high expression group (n = 89).

Association between lncRNA MEG3 expression and the clinicopathological features of BC

Subsequently, the correlation of lncRNA MEG3 expression with clinicopathological features of BC patients was shown in **Table 1**. By statistical analyses, our results indicated that low lncRNA MEG3 expression was significantly correlated with lymph node metastasis, TNM stage, and molecular subtypes ($P < 0.05$) of BC patients. However, the expression of lncRNA MEG3 was not associated with other clinicopathological factors of BC patients, including age, tumor size, histological classification, menopausal status as well as family history ($P > 0.05$). These data indicated that down-regulation of lncRNA MEG3 might play a critical role in BC progression and hormone receptor loss.

Association between lncRNA MEG3 expression and survival in BC patients

To further investigate the correlations of lncRNA MEG3 expression level with survival of BC patients, Kaplan-Meier analyses were performed. As shown in **Figure 3**, the 5-year overall survival of low lncRNA MEG3 expression group was significantly shorter than that of high lncRNA MEG3 expression group ($P < 0.05$). Moreover, the 5-year relapse-free survival of low lncRNA MEG3 expression group was also significantly shorter than that of high lncRNA MEG3 expression group (**Figure 4**, $P < 0.05$). Furthermore, in a multivariate Cox model, we found that lncRNA MEG3 expression was an independent poor prognostic factor for both 5-year overall survival (HR = 3.162, 95% CI = 1.026-9.741, $P = 0.045$, **Table 2**) and 5-year relapse-free survival (HR = 2.730, 95% CI = 1.147-6.497, $P = 0.023$, **Table 3**) in BC patients.

Discussion

LncRNAs dysregulation has been found in cancer tissues, which also functions as oncogenes or tumor suppressor genes [4]. And these dysregulated lncRNAs may support new therapeutic targets and biomarkers for cancer [3, 4]. For example, H19 is over expressed in bladder, lung, esophageal, breast and gastric cancers, and its overexpression is often correlated with poor prognosis in these cancers [14]. Meanwhile, knockdown expression of H19 reduces breast and lung cancer cell proliferation [15, 16]. In this study, we quantified lncRNA MEG3 in BC by qRT-PCR and explored its clinical relevance.

MEG3 is a ncRNA with a length of about 1.6 kb nucleotides, which locates on chromosome 14q32 [12]. It is an imprinted gene expressed from the maternal allele [12]. Although MEG3 is expressed in many human normal tissues, decreased MEG3 expression has been found in various types of human tumors, including bladder cancer, non-small cell lung cancer, cervical cancer, acute myeloid leukemia, and other can-

Table 2. Univariate and multivariate analyses for overall survival (Cox proportional hazards regression model)

Risk factor	Univariate		Multivariate	
	HR (95% CI)	*P*	HR (95% CI)	*P*
Age	2.375 (1.151, 4.904)	0.019	1.476 (0.516, 4.220)	0.468
Menopausal status	0.278 (0.135, 0.574)	0.001	0.354 (0.124, 1.007)	0.052
Histological classification	1.196 (0.519, 2.757)	0.677	1.000 (0.510, 1.961)	0.999
Family history	1.587 (0.766, 3.291)	0.674	2.342 (0.952, 5.760)	0.064
T	1.276 (0.699, 2.330)	0.428	0.971 (0.552, 1.706)	0.918
MEG3	4.343 (1.526, 12.361)	0.006	3.162 (1.026, 9.741)	0.045
LN status	14.615 (3.498, 61.067)	0.000	8.750 (1.735, 44.137)	0.009
Stage	9.273 (4.182, 20.565)	0.000	6.754 (2.382, 19.152)	0.000
Molecular subtype	1.404 (1.125, 1.753)	0.003	0.611 (0.437, 0.855)	0.004

Table 3. Univariate and multivariate analyses for relapse free survival (Cox proportional hazards regression model)

Risk factor	Univariate		Multivariate	
	HR (95% CI)	*P*	HR (95% CI)	*P*
Age	1.337 (0.766, 2.335)	0.307	1.018 (0.463, 2.242)	0.964
Menopausal status	0.541 (0.247, 1.185	0.125	0.541 (0.247, 1.185	0.125
Histological classification	0.786 (0.467, 1.323)	0.364	0.867 (0.499, 1.505)	0.611
Family history	1.033 (0.516, 2.066)	0.927	1.587 (0.766, 3.291)	0.214
T	0.994 (0.609, 1.624)	0.982	0.786 (0.489, 1.264)	0.321
MEG3	4.052 (1.818, 9.032)	0.001	2.730 (1.147, 6.497)	0.023
LN status	19,687 (6.108, 63.450)	0.000	11.941 (3.300, 43.207)	0.000
Stage	11.638 (6.161, 21.985)	0.000	5.358 (2.480, 11.575)	0.000
Molecular subtype	1.587 (1.316, 1.912)	0.000	0.725 (0.552, 0.952)	0.021

cers [7, 9, 12, 17]. Zhang et al shown MEG3 was down-regulated in cervical cancer and affected cell proliferation and apoptosis by regulating miR-21, indicating that MEG3 can be used as potential therapeutic target of cervical cancer [18]. Lu et al found that MEG3 expression was decreased in non-small cell lung cancer (NSCLC) tumor tissues compared with normal tissues, and associated with advanced pathologic stage, and tumor size, and predicted poor prognosis [19]. Furthermore, they revealed that overexpression of MEG3 not only decreased NSCLC cells proliferation and induced apoptosis *in vitro*, but also impeded tumorigenesis *in vivo*, which was dependent on upregulation of MDM2 and p53 protein levels [19]. Peng et al revealed that MEG3 decreased in gastric cancer and MEG3 decreased expression was associated with metastatic gastric cancer [20]. Furthermore, they found ectopic expression of MEG3 in human gastric cancer cells inhibited malignant phenotypes, and promoted cell apoptosis, which might be due to MEG3 sequestering oncogenic miR-181s in GC cells [20]. These studies indicated that MEG3 played a critical role in tumor progression. However, the relationship between MEG3 expression and BC development and/or progression is still unknown.

Our study was designed to investigate the expression and prognostic significance of MEG3 in BC patients. MEG3 expression was retrospectively analyzed in 257 BC patients. Results were assessed for association with clinical features and overall survival of BC patients after surgery. Prognostic values of MEG3 expression and clinical outcomes were also evaluated by Cox regression analysis. Our results showed that MEG3 expression was down-regulated in BC tissues and associated with lymph node metastasis, TNM stage and molecular subtypes. More importantly, we found that MEG3 expression was significantly

associated with relapse free survival and overall survival of BC patients. In support of this, Kaplan-Meier analysis of overall survival revealed that BC patients with decreased MEG3 expression tend to have a poorer relapse free survival and overall survival, indicating that decreased MEG3 expression is a marker of poor prognosis for overall survival of patients with BC. Multivariate Cox regression analysis proved that decreased MEG3 expression was an independent prognostic factor for BC. Although our results shown decreased expression was associated with molecular subtypes, the body of samples was not sufficient for subclass analysis. Taken together, our study demonstrated that decreased expression of MEG3 in BC is associated with more malignant subtypes and a worse prognosis implies that it could play roles of tumor suppressor gene in breast carcinogenesis.

In summary, to the best of our knowledge, the present study is the first time to report that lncRNA MEG3 expression was down-regulated in BC tissues and associated with biological aggressiveness and poor prognosis. Our study indicated that MEG3 was an independent prognostic factor of BC patients. These findings suggested that MEG3 could be a potential diagnostic and therapeutic target in patients with BC.

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Chungen Xing, Department of General Surgery, The Second Affiliated Hospital of Soochow University, 1055# Sanxiang Road, Suzhou 215004, Jiangsu, P. R. China. Tel: 86-512-6828-2030; Fax: 86-512-6828-4303; E-mail: cgxingsah@163.com

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