Int J Clin Exp Pathol 2017;10(12):11554-11562
www.ijcep.com /ISSN:1936-2625/IJCEP0067678

Original Article

MLF1IP promotes cells proliferation and apoptosis by regulating CyclinD1 in breast cancer

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Received October 21, 2017; Accepted November 8, 2017; Epub December 1, 2017; Published December 15, 2017

Abstract: Breast cancer is the most frequently diagnosed cancer and the leading causes of cancer death among females in worldwide. It is urgent to develop novel biomarkers to improve risk stratification and optimize therapy choice. In our previous study, we firstly found that MLF1IP was upregulated in breast cancer tissue compared with adjacent normal tissue and patients with high MLF1IP expression had significantly lower overall survival. However, the biological function and cellular mechanisms of MLF1IP in breast cancer is still need to be elucidated. Here, we further investigated the role of MLF1IP in breast cancer by in vivo experiments. Our results showed that the expression level of MLF1IP was associated with lymph nodes metastasis and tumor size in clinical characteristic features. By biological function experiment, we found MLF1IP is correlated with cell proliferation and apoptosis and arrest cell cycle G1 through regulating Cyclin D1. Taken together, our findings suggested that MLF1IP could contribute to the oncogenic potential of breast cancer. To the best of our knowledge, it was firstly reported that MLF1IP was involved in breast cancer. This study provided a potential new marker and a target for gene therapy in breast cancer treatment.

Keywords: Breast cancer, MLF1IP, cyclinD1

Introduction

Breast cancer is the most frequently diagnosed cancer and the leading causes of cancer death among females in worldwide [1]. Breast cancer alone accounts for 25% of all cancer and 15% of all cancer deaths among females. There are estimated 1.7 million cases and 521,900 deaths in 2012 [2]. During the 1990s, the incidence rate of breast cancer rose about 30% in Western countries due to the use of menopausal hormone therapy and spread of screening [3]. Breast cancer is a clinicopathologically heterogeneous disease with a different outcome that is not accordance with histological features. Molecular variances lead to differences in clinical behavior and response to therapy even in histologically similar tumors [4]. Therefore, it is urgent to develop novel biomarkers to improve risk stratification and optimize therapy choice.

The myelodysplasia leukemia factor 1-interacting protein (MLF1LP, also called KLIP1 and CENP-50) was first identified as a novel protein that is associated with MLF1 (myelodysplasia leukemia factor 1) [5]. The mRNA of MLF1IP encodes a 47 kDa protein, including two nuclear receptor binding motifs, two leucine zippers and a number of potential phosphorylation sites [5]. It was reported that MLF1LP played an important role in cells mitosis [6]. Recently, Pan et al. demonstrated MLF1IP played an important role in transcriptional regulation, indicating that MLF1IP may be a new member of cellular transcriptional repressors [7]. Zhang's study also showed MLF1IP significantly promoted prostate cancer cell proliferation and colony formation and significantly inhibited apoptosis [8]. But the biological function of MLF1IP is still unknown in breast cancer.

In our previous study, we firstly found that MLF1IP was upregulated in breast cancer tissue compared with adjacent normal tissue by real-time reverse transcription-polymerase chain reaction (RT-qPCR) and patients with high MLF1IP expression had significantly lower overall survival [9]. However, the biological function and cellular mechanisms of MLF1IP in breast cancer is still need to be elucidated. Here, we

Table 1. MLF1IP-ShRNA sequence

ShRNA names	TopStrand sequences	Bottom Strand sequences
shRNA1	CCCAGGTATGAGCTATAATAATTCAAGAGATTATTATAGCTCATACCTGGGTTTTTT	AAAAAACCCAGGTATGAGCTATAATAATCTCTTGAATTATTATAGCTCATACCTGGG
shRNA2	GCTCAAGAACCAAACGTAAAGTTCAAGAGACTTTACGTTTGGTTCTTGAGCTTTTTT	AAAAAAGCTCAAGAACCAAACGTAAAGTCTCTTGAACTTTACGTTTGGTTCTTGAGC
shRNA3	GCGATTTCTCCTGAATTTACATTCAAGAGATGTAAATTCAGGAGAAATCGCTTTTTT	AAAAAAGCGATTTCTCCTGAATTTACATCTCTTGAATGTAAATTCAGGAGAAATCGC
shRNA4	GGACTTAGTACCCATGCTTTATTCAAGAGATAAAGCATGGGTACTAAGTCCTTTTTT	AAAAAAGGACTTAGTACCCATGCTTTATCTCTTGAATAAAGCATGGGTACTAAGTCC

further investigated the role of MLF1IP in breast cancer by *in vivo* experiments.

Methods

Tissue samples

A total of 66 patients who were diagnosed with luminal breast cancer and treated with surgery at the Affiliated Hospital of Wenzhou Medical University from September 1, 2016 to November 3, 2016 were enrolled in this study. No prior treatments (including chemotherapy, endocrine therapy or radiotherapy) were conducted before the breast cancer resection surgery. All tumor tissues were diagnosis as invasive ductal carcinoma, whose results were confirmed by two pathologists. This study was approved by the Ethics Committee of the First Affiliated Hospital of Wenzhou Medical University. All patients provided written informed consent in order to participate in this study.

Immunohistochemistry and immunohistochemical assessment

Immunohistochemical studies on MLF1IP were performed on formalin-fixed, paraffin-embedded tissue sections obtained from the aforementioned patients with luminal breast cancer and were performed according to the standard procedures. Sections were cut at a thickness of 4 μm and heated in a 70°C oven. Briefly, tissue sections were deparaffinized, dehydrated and boiled in 0.01 mol/l sodium citrate buffer (pH 6.0) in a microwave oven for 10 min for antigen epitope retrieval. Endogenous peroxidase was blocked with 0.3% hydrogen peroxide for 15 min. Then, the sections were blocked for 30 min using 10% normal goat serum and were separately incubated with primary antibodies directed against MLF1IP (HPA022048, 1:1000 dilution; Sigma. USA) at 4°C overnight. After washing, the sections were incubated for 60 min with secondary antibody (HRP) (Santa Cruz, USA) at 37°C. The complex was visualized with diaminobenzidine (DAB) and countersigned with hematoxylin. The sections were then dehydrated in a graded series of alcohol, cleared in xylene, mounted onto glass slides.

MLF1IP staining criteria were scored as follows: according to the expression of the area, 0% of staining was recorded as 0; 1~10% of staining was recorded as 1; 11-25% of staining was recorded as 2; 26-50% of staining was recorded as 3; and >50% of staining was recorded as 4. According to the expression extent, tumor cells without staining was recorded as 0; tumor cells with low staining was recorded as 1; medium staining was recorded as 2; and extensive staining was recorded as 3. Combined expression area and extent, total scores of 0 to 2 were considered low expression and scores of 3 to 6 considered high expression for further analysis.

Cell cultures and transfected

Cell culture breast cell lines MCF-7, T47D and MDA-MB-231 were propagated in RPMI-1640 supplemented with 10% fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA, USA). All cell lines were maintained at the cell culture incubator with 37°C and 5% CO_2. All cell lines were provided by American type culture collection (ATCC). In this study, the lentivirus vectors for lentivirus-mediated downregulation of MLF1IP were purchased from GenePharma (Shanghai, China). MLF1IP shRNA encoding lentiviruses and their control lentiviruses were used to infect MCF-7, T47D and MDA-MB-231. Quantitative real-time PCR (qRT-PCR) assay were used to monitor infection efficiency when infected cells were collected 48 h after lentivi-

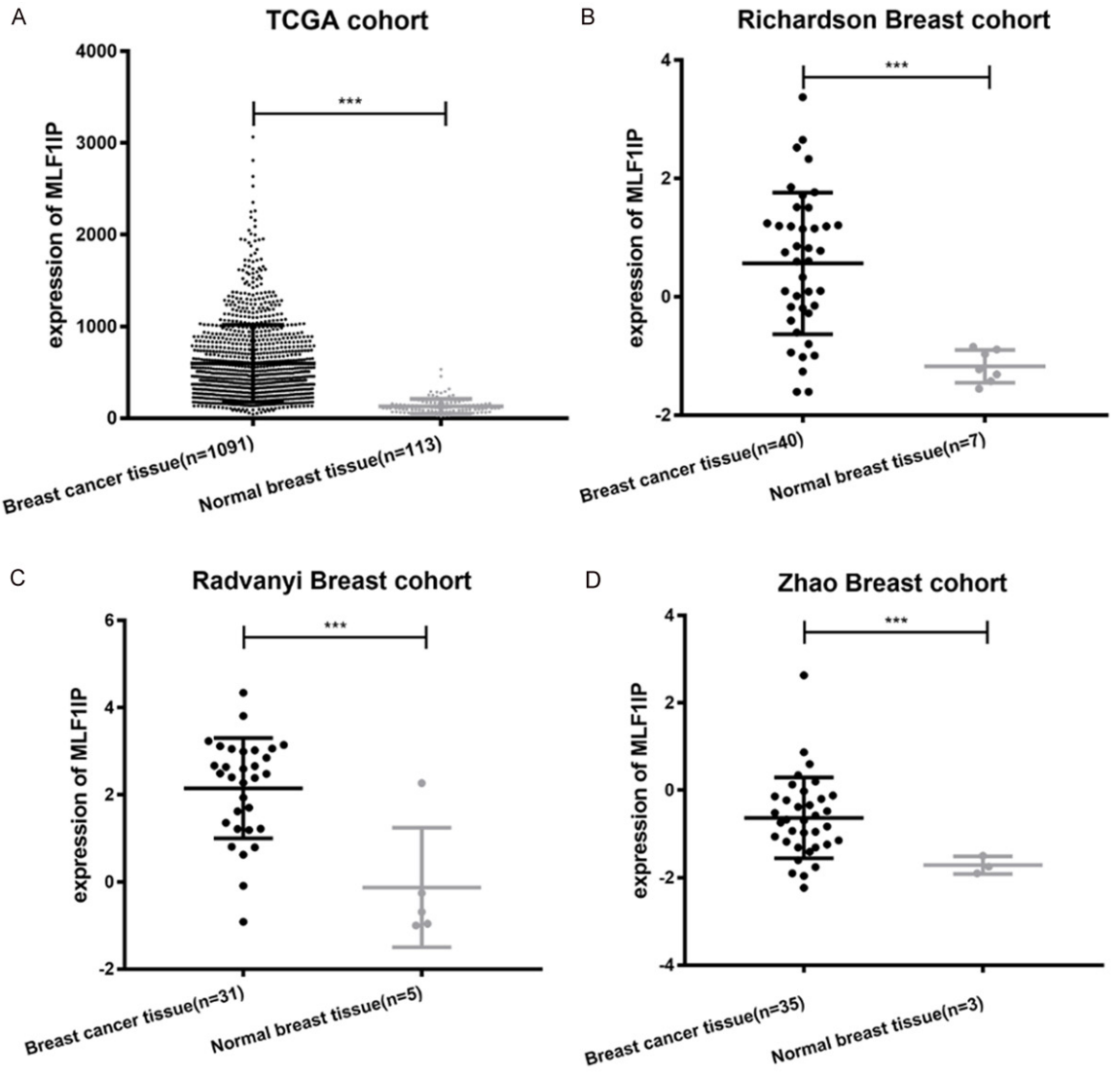

Figure 1. MLF1IP expression in TCGA and Oncomine database. A. MLF1IP expression level of breast cancer in TCGA database. B. MLF1IP expression level of breast cancer in Richardson Cohort from Oncomine database. C. MLF1IP expression level of breast cancer in Radvanyi Cohort from Oncomine database. D. MLF1IP expression level of breast cancer in Zhao Cohort from Oncomine database. ****P*<0.001 in comparison with the normal breast tissues using Student's t-test.

rus infection. MLF1IP-ShRNA sequences were showed in **Table 1**.

Cell viability assay

Cell viability was measured with CCK-8 kit, followed the manufacturer. Briefly, cancer cells seeded in 96-well plates were transfected with lentivirus. All cells were then incubated at 37°C for consecutive 5 days. CCK-8 solution was added and incubated with cancer cells for 2 h. Relative absorbance was measured at OD 450 by SpectraMax 5 microplate reader (Molecular Devices).

RNA isolation and real-time reverse transcription-polymerase chain reaction

Total RNA was isolated from tissues or cell lines using TRIzol reagent (Invitrogen) according to manufacturer's instructions. Real-time PCR analysis was performed by The Applied Biosystems QuantStudio 5 Real-Time PCR System (Applied Biosystems, Foster City, CA) with the THUNDERBIRD SYBR qPCR Mix (Toyobo) according to manufacturer's instructions. The GAPDH was used as internal control. The relative expression levels were calculated by the

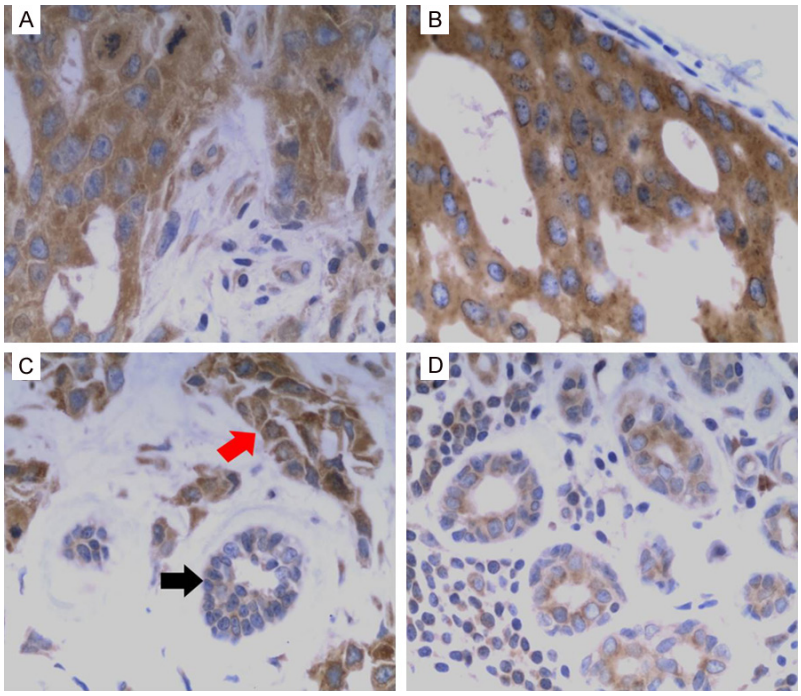

Figure 2. Immunohistochemical staining of MLF1IP proteins in breast cancer and adjacent normal tissues. A, B. High MLF1IP immunohistochemical staining was shown in breast cancer tissues. C. MLF1IP immunohistochemical staining was shown in breast cancer (red arrow) and adjacent normal tissues (black arrow). D. Low MLF1IP immunohistochemical staining was shown in adjacent normal tissues as negative control.

equation $2^{-\Delta\Delta CT}$. The primer sequences for PCR are as follows: MLF1IP forward: 5'-CAGAAGGAATGAAAACCAGTGACA-3' and reverse: 5'-ATGTGGCGATGGCTGCCTTACA-3' GAPDH forward: 5'-GTCTCCTCTGACTTCAACAGCG-3' and reverse 5'-ACCACCCTGTTGCTGTAGCCAA-3'.

Cell cycle and cell apoptosis assays

Propidium iodide (PI) staining assay was used to analyze the cell cycle distribution. After transfected for 48 h, cancer cells were harvested and fixed with 70% ethanol, followed by centrifugation (3000 rpm, 5 min), incubation with 100 mg/mL RNase in PBS for 30 min at 37°C, and then staining with 50 mg/mL PI in PBS. The cell cycle distributions were analyzed by a Cell Lab Quanta SC flow cytometer (Beckman Coulter, USA).

The Annexin V-FITC Apoptosis Detection Kit (BioVision) was used for the apoptosis analysis. Cells (5 × 105) were transfected after 48 h. After resuspended in 500 ml binding buffer, cells were incubated with Annexin V-fluorescein isothiocyanate (FITC; 5 ml) and PI (5 ml). After 30 min incubation, cells were analyzed by fluorescence-activated cell sorting (FACS) by flow cytometer (Becton Dickinson).

Protein extraction and Western blot analysis

Treated cells were lysed in RIPA lysis buffer (Beyotime, Shanghai, China). An equal amount of protein of about 20 μg was separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred onto the polyvinylidene fluoride membrane. After blocking with 5% skimmed milk, the polyvinylidene fluoride membrane was incubated with anti-ARAP3 antibody (Abcam, USA). After washing three times with Tris-buffered saline and Tween 20, the membrane was incubated with horseradish peroxidase-linked secondary anti-goat immunoglobulin G antibody (Abcam) at room temperature for 1.5 h. GAPDH protein, detected using an anti-GAPDH antibody (Abcam), was used for control.

Statistical analysis

Student's t-test was used to evaluate significant differences between the two groups in gene expression levels and cellular experiments. Clinicopathological characteristics were evaluated using the Chi-square test or Wilcoxon test as appropriate. Data was presented as the mean ± standard deviation of three independent experiments. *P* values less than 0.05 were considered statistically significant. All statistical analyses were performed using SPSS version 22.0 (Chicago, IL).

Results

MLF1IP was up-regulated in breast cancer

In our previous study, the expression of MLF1IP mRNA was detected in 15 paired breast cancer tissues and adjacent non-cancerous tissues by qRT-PCR [9]. The result showed that MLF1IP mRNA was significantly up-regulated in breast cancer tissues compared with the adjacent nor-

Table 2. The relationship between MLF1IP protein expression and clinicopathologic characteristics in breast cancer patients of IHC (n=66)

Characteristic	Low MLF1IP (%)	High MLF1IP (%)	X^2	*P*
Age			0.322	0.570
<60	35 (85.4%)	20 (80.0%)		
≥60	6 (14.6%)	5 (20.0%)		
Menopause			0.142	0.706
Pre-menopause	20 (48.8%)	11 (44.0%)		
Post-menopause	21 (51.2%)	14 (56.0%)		
Tumor size			4.151	0.042
≤2 cm	22 (53.7%)	7 (28.0)		
>2 cm	19 (46.3%)	18 (72.0%)		
Lymph-node metastasis			6.068	0.014
None	29 (70.7%)	10 (40.0%)		
Present	12 (29.3%)	15 (60.0%)		
Clinical stage			1.319	0.251
I-II	33 (80.5%)	17 (68.0%)		
III-IV	8 (19.5%)	8 (32.0%)		
Progesterone receptor			10.677	<0.001
Negative	6 (14.6%)	13 (52.0%)		
Positive	35 (85.4%)	12 (48.0%)		
HER2 status			0.618	0.432
Negative	30 (73.5%)	16 (64.0%)		
Positive	11 (26.8%)	9 (36.0%)		

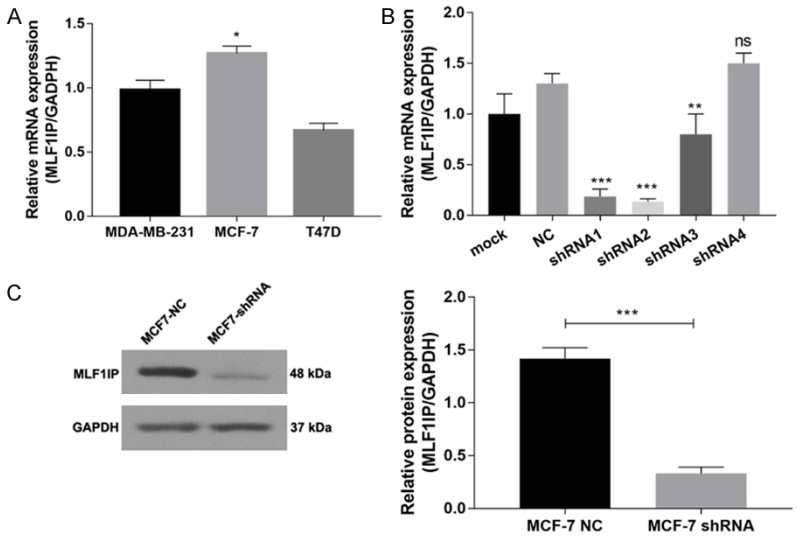

Figure 3. Target cell selection and lentivirus transfection efficiency. A. The relative expression of MLF1IP in MDA-MB-213, MCF-7, T47D. Compared to the other cell lines, MCF-7 cells exhibited the highest MLF1IP expression. B. The relative expression of MFL1IP in six groups. The expression of MLF1IP in shRNA2 group was lowest, compared with other groups. C. The relative expression of MLF1IP in protein level. Left: MLF1IP expression is knocked down after transfected shRNA2 measured by Western blot. Right: Relative quantification of MLF1IP expression. **P*<0.05, ***P*<0.01, ****P*<0.001 in comparison with the NC group using Student's t-test.

mal tissues (*P*<0.01). Mining published transcriptome data was of low cost and feasible for exploring gene expression. To validate the fidelity of our previous study, we detected the gene expression in The Cancer Genome Atlas (TCGA) cohort containing 1091 breast cancer samples and 113 normal breast tissue samples [10]. Result showed that MLF1IP was markedly up-regulated in breast cancer tissues compared with the adjacent normal tissues (*P*<0.001, **Figure 1A**). Moreover, different cohorts in Oncomine database had been further validated that MLF1IP was up-regulated significantly in breast cancer tissues (*P*< 0.001, **Figure 1B-D**) [11-13]. Immunohistochemical studies were also carried out to detect MLF1IP protein expression patterns in 66 breast cancer tissues and their corresponding normal tissues. MLF1IP expression was observed in the cytoplasmic compartments of tumor cells, while normal breast tissues exhibited negative or weak expression (**Figure 2**).

MLF1IP was associated with tumor size and lymph node metastasis

To analyze whether MLF1IP was associated with the occurrence and progression of breast cancer, we investigated the association between clinical features and MLF1IP expression level. In our study, patients were categorized as low or high group depending on MLF1IP expression level. The results revealed tumor size (*P*=0.042), lym-

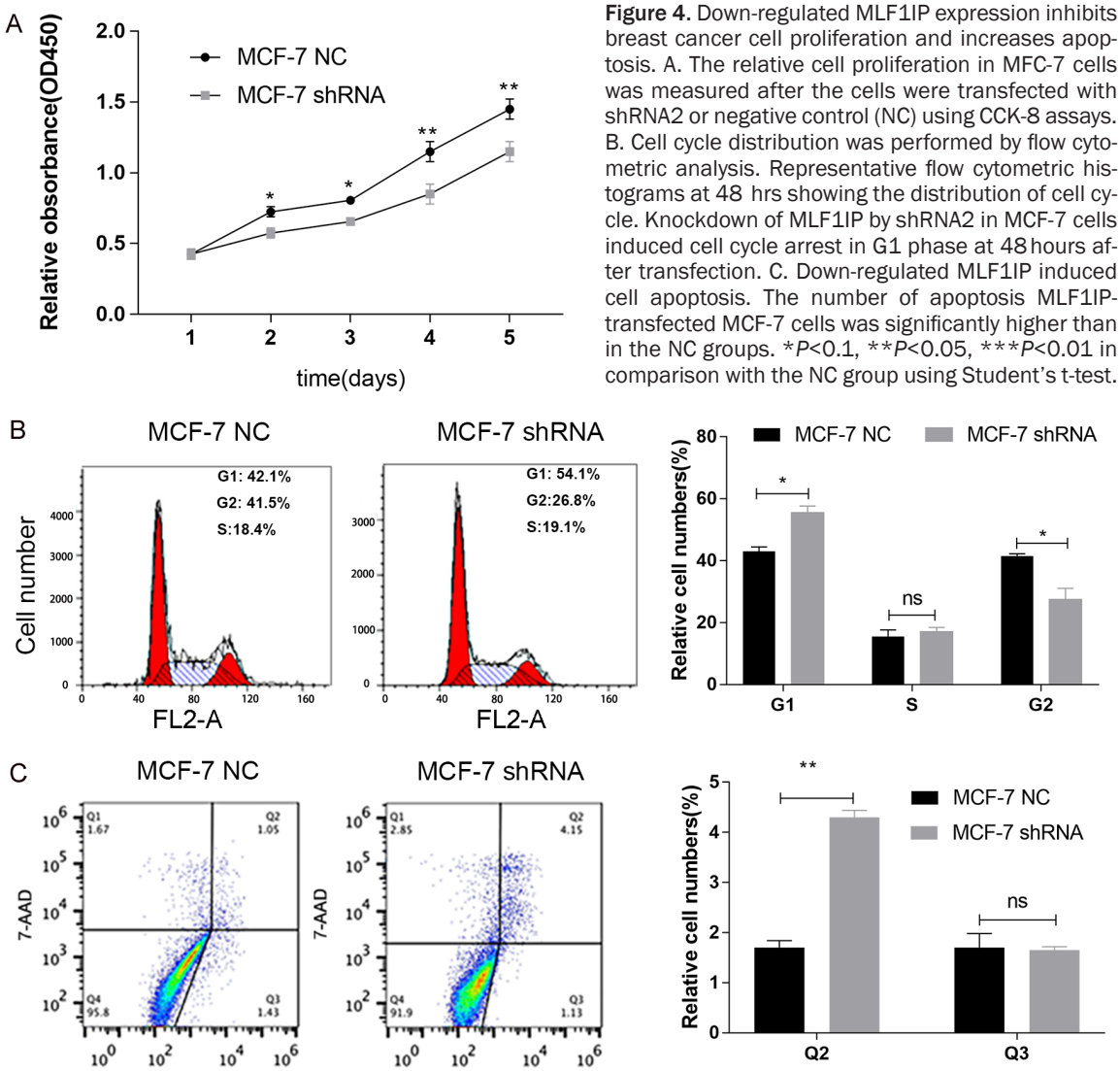

Figure 4. Down-regulated MLF1IP expression inhibits breast cancer cell proliferation and increases apoptosis. A. The relative cell proliferation in MFC-7 cells was measured after the cells were transfected with shRNA2 or negative control (NC) using CCK-8 assays. B. Cell cycle distribution was performed by flow cytometric analysis. Representative flow cytometric histograms at 48 hrs showing the distribution of cell cycle. Knockdown of MLF1IP by shRNA2 in MCF-7 cells induced cell cycle arrest in G1 phase at 48 hours after transfection. C. Down-regulated MLF1IP induced cell apoptosis. The number of apoptosis MLF1IP-transfected MCF-7 cells was significantly higher than in the NC groups. **P*<0.1, ***P*<0.05, ****P*<0.01 in comparison with the NC group using Student's t-test.

ph node metastasis (*P*=0.014) and progesterone receptor (*P*<0.001) were significant related with the expression of MLF1IP positively (**Table 2**). Our data indicated that MLF1IP was involved in the progress of breast cancer and it might act as an oncogene.

Regulation expression of MLF1IP in breast cancer cell lines MCF-7

In order to investigate the biological function of MLF1IP in breast cancer cell lines, we compared the expression levels of MLF1IP in MDA-MB-231, MCF-7, and T47D by qRT-PCR (**Figure 3A**). The result showed the relative expression level of MLF1IP in multiple breast cancer cell lines, and the MCF-7 cells that have relatively high levels of MLF1IP were chosen for further knockdown assays (*P*<0.05, **Figure 3A**). Then we designed four specific short hairpin RNA (shRNA) and transfected MCF-7 cells permanently with pLV-shRNA lentiviral. Controls were infected with empty lentivirus particles. The qRT-PCR assays demonstrated that shRNA2 could significantly knock down expression of MLF1IP (*P*<0.001, **Figure 3B**). Western blotting also showed that the protein expression level of MLF1IP was down-regulated by transfecting pLV-shRNA2 compared with negative control (*P*<0.001, **Figure 3C**).

Down-regulated MLF1IP expression inhibits breast cancer cell proliferation and increases apoptosis

In order to investigate the effect of MLF1IP on proliferation of breast cancer cell lines, we performed proliferation assays. The amount of cell

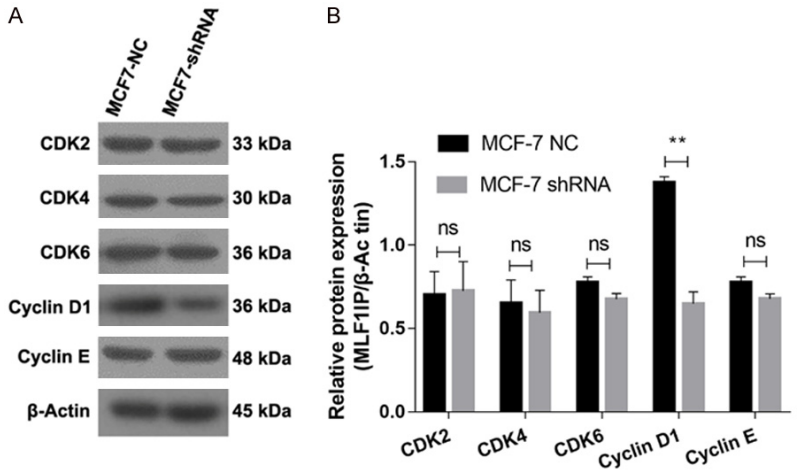

Figure 5. Effect of downregulation of MLF1IP on protein expression level of cell cycle in MCF-7 cells. A. Total cellular protein were extracted at 48 hours after transduction and determined by Western blot analysis using antibodies against CDK2, CDK4, CDK6, Cyclin D1, Cyclin E and β-Actin as a loading control. B. Relative quantification of MLF1IP expression. Down-regulated MLF1IP significant knocked down Cyclin D1 expression. $^{**}P<0.01$ in comparison with the NC group using Student's t-test.

proliferation was determined using the CCK-8 assay once daily for 5 days. In our study, down-regulated MLF1IP inhibited MCF-7 cells proliferation in a time-dependent manner compared with the NC group ($P<0.01$, **Figure 4A**). To further explore the growth suppression effect of MLF1IP on MCF-7 cells, we carried out cell cycle distribution analysis by flow cytometry at 48 hours after transfected. The results showed that down-regulated MLF1IP induced cell cycle arrest in G1 phase in MCF-7 cells ($P<0.05$, **Figure 4B**). Compared with the NC group, the percentage of G1 phase in the shRNA2 group was increased by 12.0%, while the percentage of G2 phase in the shRNA2 group was decreased by 14.7% ($P<0.05$). The result demonstrated that MFL1IP silencing may induce cell cycle arrest at G1 phase. To determine whether MLF1IP play an important role in apoptosis of MCF-7, we evaluated the rate of cellular apoptosis by flow cytometry. The results showed that the fraction of apoptotic cells was significantly increased among the shRNA2-infected cells compared with the NC-treated cells ($P<0.01$, **Figure 4C**). These results suggested that MLF1IP was a potential oncogene in breast cancer.

MLF1IP promotes cells proliferation by regulating CyclinD1 in breast cancer

To investigate the underlying mechanism inducing cell cycle arrest, we tested the effect of MLF1IP knockdown on a series of cell cycle-related molecules. Western blotting analysis revealed that knockdown of MLF1IP decreased cyclin D1 protein level in MFC-7 lines ($P<0.01$, **Figure 5**). However, we did not observe remarkable changes in CDK2, CDK4, CDK6 and cyclin E expression (**Figure 5**). These results suggest that MLF1IP affects breast cancer cell growth by regulating cyclin D1 expression.

Discussion

Breast cancer is the most frequently diagnosed cancer and is the sixth leading cause of cancer-related death in China [14]. So it is urgent to develop precise approaches to diagnose and treat breast cancer. As sequencing technology developing, it provided a new approach to find key molecular biomarkers to guide diagnosis, targeted therapy and predicting prognosis [15, 16]. For instance, breast cancer patients with HER-2 genes overexpression and/or amplified are associated with a more aggressive phenotype and shorter survival time. While the widespread utilization of HER-2 targeted agents such as Trastuzumab had dramatically improved prognosis of those patients [17, 18]. Although we have found many oncogenes and made a great progression, there is still unknown mechanism of occurrence and progression of breast cancer.

The MLF1IP gene encodes a 46-kDa nuclear-localizing transcription suppressor protein that has been previously associated with malignancy [19]. In 2005, Hanissian et al. found that MLF1IP play an important role in glioblastoma tumor development [20]. Another research found that MLF1IP is up-regulated in familial colorectal cancer when compared with normal tissue [21]. What's more, MLF1IP could significantly promote prostate cancer cell proliferation and inhibited apoptosis in prostate cell lines, which is accordance with expression level change in tumor tissue compared with normal tissue [8]. In our previous study, we also dem-

onstrated that MLF1IP is correlated with progression and prognosis in breast cancer [9]. Here, we studied the biological function and cellular mechanisms of MLF1IP in breast cancer. Our results showed that the expression level of MLF1IP was associated with lymph nodes metastasis and tumor size in clinical characteristic features. By biological function experiment, we found MLF1IP is correlated with cell proliferation and apoptosis and arrest cell cycle G1 through regulating Cyclin D1. Taken together, our findings suggested that MLF1IP could contribute to the oncogenic potential of breast cancer. To the best of our knowledge, it was firstly reported that MLF1IP was involved in breast cancer. This study provided a potential new marker and a target for gene therapy in breast cancer treatment.

In the present study, we also found that knockdown of MLF1IP decreased cyclin D1 protein level. Cyclin D1 is a cell cycle regulator which playing an important role in regulating progression from the G1 to S phase by formatting active enzyme complexes with cyclin-dependent kinases, CDK4 and CKD6 [22].

The cyclin D1 was frequently overexpressed in a wide range of cancers [23]. The nuclear accumulation of cyclin D1 induced uncontrolled proliferation in normal human cells, which could facilitate the development of invasive cancer [24]. In addition, an increasing number of reports have shown that suppression of cyclin D1 reduced the proliferation of cell cells while inducing apoptosis in a large number of cancers [25].

Our study still has several limitations. On the one hand, we found the association between MLF1IP and Cyclin D1, but the mechanism of interaction is needed further to investigate. What's more, although we demonstrated the biological function of MLF1IP in vitro, the in vivo experiments still needed to be performed to validate the effect of MLF1IP.

Acknowledgements

This study was funded by National Natural Science Foundation of China (No.81372380, N0.81572291) and Natural Science Foundation of Zhejiang province (LY18H160053, LY17-H160053 and LY18H160053) and the Science And Technology Project of Wenzhou (Y20160-036).

Disclosure of conflict of interest

None.

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