

Original Article

LncRNA MALAT1 promotes relapse of breast cancer patients with postoperative fever

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Abstract: Postoperative fever is prevalent in many breast cancer patients. Some retrospective studies proposed that postoperative fever might also be considered as a rapid rough indicator for the poor prognosis of breast cancer patients. This study aims to explore the possible molecular mechanisms underlying the relapse of breast cancer patients with early postoperative fever. Our results indicated plasma levels of lncRNA MALAT1 were elevated in breast cancer patients with early postoperative fever and were associated with RFS. Lipopolysaccharide (LPS) was able to induce fever and systemic inflammatory responses in 4T1 xenograft mice, and promote lung metastasis. But after knocking down lncRNA MALAT1, the inflammatory responses and metastasis of lung were significantly reduced. Moreover, after knocking down lncRNA MALAT1 in the 4T1 cells, TNF- α level in the supernatants was sharply decreased, and the invasion and migration induced by LPS was also weakened. Cumulatively, our data indicates that MALAT1 is closely related to recurrence and metastasis of breast cancer patients with early postoperative fever.

Keywords: MALAT1, breast cancer, postoperative fever, recurrence, metastasis

Introduction

Breast cancer is the most common malignancy among women in developed countries, accounting for 27% of total cancer cases and 14% of total cancer-related deaths in 2012 [1]. Although delicate surgical ablation, effective chemotherapy and accurate radiotherapy may eradicate the visible primary tumor, metastasis and recurrence still occur frequently in some breast cancer patients, which eventually leads to the death [2]. Metastasis is not only regulated by intrinsic genetic changes in breast cancer cells but also depends upon tumor micro-environment [3]. Several studies have demonstrated that tumor-associated chronic inflammation is a hallmark of cancer and it also promotes disease progression and metastasis [4].

Postoperative fever refers to core temperature rises in patients after surgery. Early postoperative fever (>38°C in the first 72 h) is rarely caused by an infection and usually believed to

be one of the host responses to surgery [5]. As a complex systemic defense reaction in the host, postoperative fever may result from the release of specific cytokines induced by infection, injury, surgery and general anesthetic trauma [6-8]. An early retrospective study in 1987 supported the association between postoperative fever and prognosis in 387 cases of breast cancer patients [9]. *Lu J, et al* also found that postoperative fever may contribute to relapse of node-negative breast cancer patients and could serve as a negative indicator of disease relapse [10].

However, the exact mechanism by which the postoperative fever leads to disease relapse and progression is remained unclear. Some relevant reports suggested that elevated expressions of pro-inflammatory cytokines, such as IL-6 and TNF- α , may exacerbate the pre-existing subclinical lesions and thus promote recurrence and death in patients with postoperative fever [11-13]. Interestingly, lncRNA metastasis-

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Table 1. Summary of breast cancer patient's characteristics and its association with postoperative fever

Variable	Univariate analysis, n (%)		P value
	Fever (n = 61)	Non-Fever (n = 197)	
Mean Age at Diagnosis (year, x ± SD)	51.0±11.3	50.4±10.8	0.627
Histologic type			
DCIS/DCIS with microinvasive	3 (10.7)	25 (89.3)	0.088
Ductal invasive cancer	51 (26.7)	140 (73.3)	
Others invasive cancer	7 (17.9)	32 (82.1)	0.251
Type of Surgery			
BCT	3 (27.3)	8 (72.7)	0.772
Mastectomy	58 (23.5)	189 (76.5)	
Axillary lymph node status			
No	32 (19.6)	131 (80.4)	0.047
Yes	29 (30.5)	66 (69.5)	
No of positive lymph nodes			
1-3	16 (25.0)	48 (75.0)	0.093
≥4	13 (41.9)	18 (58.1)	
ER Status			
Positive	37 (14.34)	135 (52.33)	0.254
Negative	24 (22.8)	62 (77.2)	
PR Status			
Positive	34 (20.6)	131 (79.4)	0.126
Negative	27 (29.0)	66 (71)	
HER-2 Status			
Positive	17 (27.9)	44 (72.1)	0.374
Negative	44 (22.3)	153 (77.7)	
Ki-67 Index			
≥30%	45 (27.3)	120 (72.7)	0.068
<30%	16 (17.2)	77 (82.8)	
Relapse events			
Yes	6 (54.5)	5 (45.5)	0.014
No	55 (22.3)	192 (77.7)	

associated lung adenocarcinoma transcript 1 (MALAT1) was also identified as an important pro-inflammatory factor, which regulated hyperglycemia-induced inflammatory process in the endothelial cells [14]. And it was first recognized as a biomarker to predict metastasis and survival in early-stage non-small cell lung cancer [15]. More important, Mao YF et al observed that the MALAT1 upregulation plays an important role in breast cancer development, and serum MALAT1 levels may be of great value in diagnosis in breast cancer [16].

Therefore, the present study sought to evaluate whether MALAT1 promoted the relapse of

patients with postoperative fever and to identify the underlying mechanisms involved.

Materials and methods

Patient population

Two hundred fifty-eight cases of consecutive patients with primary breast carcinoma admitted to the Third Hospital of Nanchang between June 15, 2014 to December 15, 2015 were included in the present study. Patients with initial distant metastases or systemic infectious diseases, systemic immunological disease, or serious liver or renal disease at the time of surgery were excluded from the study. Ethical approval was obtained from the Third Hospital of Nanchang, and informed consent was obtained from all patients prior to sample examination. The conventional clinicopathological data and treatment protocols were carefully documented. All patients were treated according to the guidelines or recommendations for the clinical diagnosis and treatment of breast cancer.

The body temperature was measured with an oral thermometer three times a day for each patient. Consistent with previous relevant reports [6, 10, 17], early postoperative fever was defined as one

oral temperature measured at 38°C in the 3 days after surgery. According to the postoperative oral temperature, the patients were stratified into two groups as follows: the fever group and the non-fever group. The clinicopathological characteristics of both groups were listed in **Table 1**.

Clinical follow-up data were available for all the patients. Personal contact with the patient were carried out in the Third Hospital of Nanchang every 3 months during the first two years and every 6 months during the next three years. The follow-up period cut-off was June 15, 2017. Release-free survival (RFS) was defined

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as the time from the diagnosis of breast cancer to the first local, regional or distant recurrence.

Measurement of serum IL-6 and TNF- α

For each patient, 5 mL of peripheral blood was collected within 12 hours after surgery. Then, the plasma samples were kept frozen at -80°C until biochemical assessment. The concentrations of cytokines in postoperative plasma samples and cell culture supernatants were determined by an ELISA Kit for human IL-6 and TNF- α (Elabscience Biotechnology Co. Ltd, China) according to the manufacturer's instructions.

RNA isolation and qRT-PCR

Total RNA was extracted from plasma samples or 4T1 mammary carcinoma cells using TRIzol[®] reagent according to the manufacturer's instructions (Invitrogen, Paisley, UK) and was stored at -80°C until use. The expression of MALAT1 was determined by quantitative reverse transcription-polymerase chain reaction (qRT-PCR) using the SYBR[®]Green (TaKaRa) dye detection method on an ABI StepOne PCR instrument. The primers were designed as follows: MALAT1 forward: 5'-CTTAAGCGCAGCGCCATTTT-3'; reverse: 5'-CCTCCAAAC CCAAGACCAA-3'; GAPDH forward: 5'-AATCCCATCACCATCTCCAG-3'; reverse: 5'-GAGCCCCAGCCTTCCTCCAT-3'. The relative expression of MALAT1 was calculated by the qRT-PCR method using the GAPDH mRNA as an internal control.

Cell culture

The 4T1 mammary carcinoma cell line was purchased from the Cell Bank of Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). 4T1 is a highly metastatic breast cancer cell line derived from a spontaneously arising BALB/c mammary tumor. The 4T1 mammary carcinoma cell line was maintained in RPMI-1640 medium supplemented with 10% FBS, 1% Glutamax-1 and 1% penicillin-streptomycin. Cells were routinely cultured in a humidified incubator with 5% CO_2 at 37°C .

For Lipopolysaccharide (LPS) or IL-6 treatment, the cells were treated with LPS (0.5 $\mu\text{g}/\text{ml}$) or IL-6 (1 ng/ml or 10 ng/ml) after reaching 60-70% confluence in six-well plates. Both the LPS and IL-6 were dissolved in phosphate-buffered saline (PBS).

Cell transfection assays and plasmid preparation

Cell transfections were performed using the Lipofectamine 2000 kit (Invitrogen) according to the manufacturer's instructions. To knock-down MALAT1, three siRNA oligonucleotides targeting MALAT1 and the negative control were purchased from GenePharma (Shanghai, China) and were transfected into 4T1 cells. The final MALAT1 and negative control (NC) siRNA concentrations for transfections were 50 nM.

The most effective sequence was selected by RT-qPCR and was used to construct a MALAT1-shRNA lentivirus (MALAT1-sh) (GenePharma, Shanghai, China). All siRNA sequences are listed in [Table S1](#). After transfecting the vector into 4T1 cell, 1 $\mu\text{g}/\text{ml}$ puromycin (Sigma-Aldrich) was added into the culture medium to obtain cell clones that stably express the shRNA. Then, the cells were cultured for an additional 48 hours before LPS treatment.

Transwell invasion assays

Transwell (24-well) chambers (Costar, Cambridge, MA, USA) were used to evaluate cell invasion. For the invasion assay, 1×10^5 4T1 cells of different groups were suspended in 200 μl serum-free medium and added into BD BioCoat Matrigel Invasion Chambers (a pore size of 8 μm ; BD Biosciences). The lower chamber was filled with RPMI-1640 medium containing 10% FBS. The chamber was incubated for 24 h in 5% CO_2 at 37°C . Finally, the attached cells in the lower section were stained with hematoxylin and eosin (H&E) and counted using light microscopy.

Western blot analysis

Cells were lysed in RIPA buffer containing fresh protease and phosphatase inhibitor cocktails (Sigma). Protein was separated by 10% SDS-PAGE and was transferred to PVDF membranes. The membranes were blocked in 2% skim milk and were incubated with anti-vimentin (1:1000, Abcam, USA) and anti-MMP-9 (1:1000, Wanlei, China) overnight at 4°C . Anti-GAPDH antibody (1:1000, Proteintech, USA) was used as an endogenous control and was incubated overnight at 4°C . Then, the membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (1:1000 dilution, Abcam, USA) for 2 h at room temperature.

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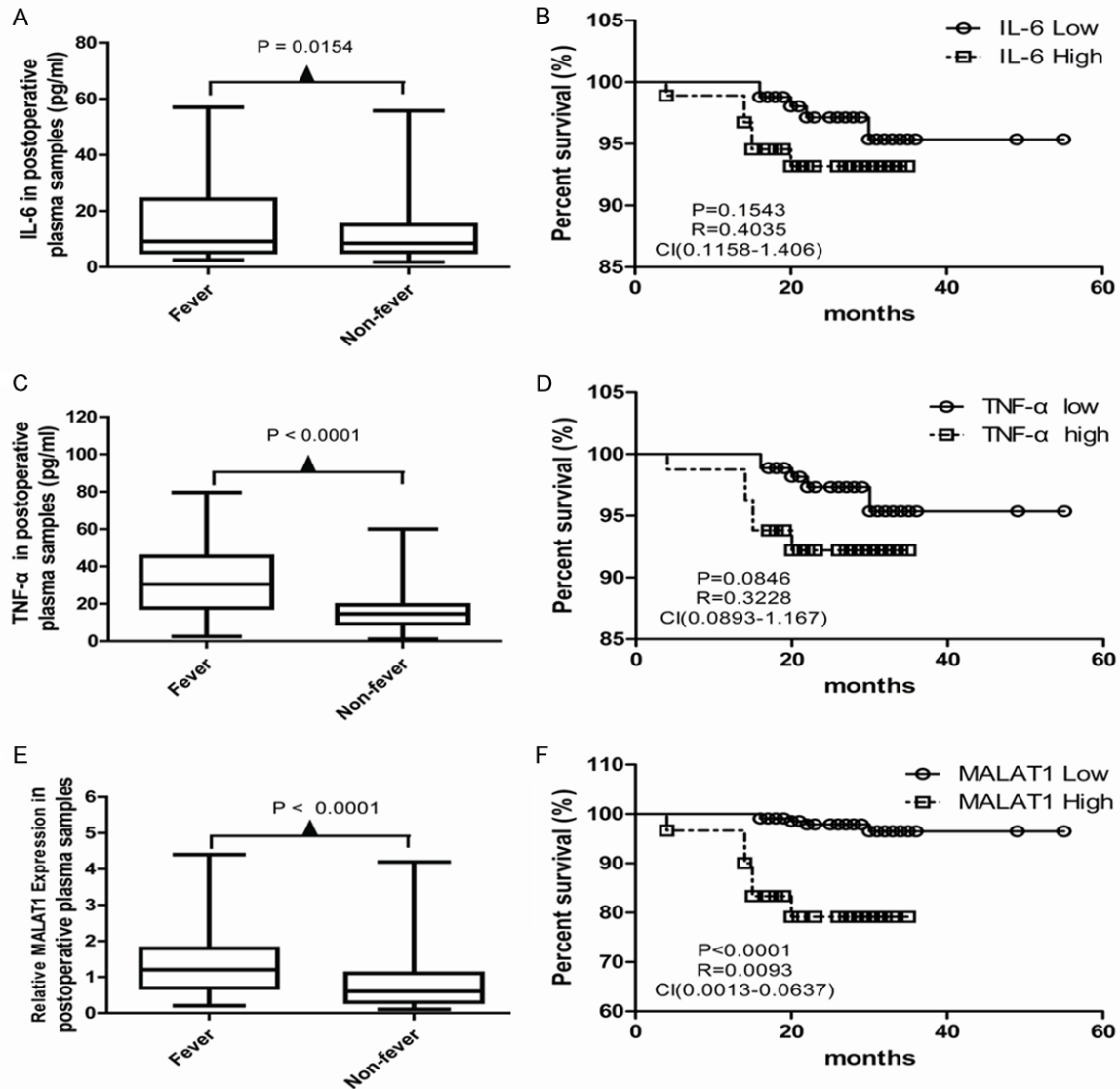


Figure 1. The association among the pro-inflammatory cytokines, postoperative fever and the prognosis of breast cancer patients. Plasma IL-6 (A), TNF- α (C) and MALAT1 (E) levels in the fever group were significantly higher than those in the non-fever group (P = 0.0154, <0.001, <0.001). Kaplan-Meier analysis of the RFS of the patients with low IL-6 expression and high IL-6 expression, (B) with low TNF- α expression and high TNF- α expression (D), and with high MALAT1 expression and low MALAT1 expression (F) in 258 primary breast cancer patients (Log-rank test P = 0.1543, 0.0846, and P<0.001, respectively).

The relative band density was determined using the Tanon 5200 Multifunctional Imaging System (Beijing, China) with the ECL Western Blotting Substrate Kit (Millipore, Billerica, MA, USA).

Mouse model of LPS-induced sepsis

Female BALB/c mice (6-8 week old) were purchased from the Laboratory Animal Research Center of the Medical college of Nanchang University and were kept under sterile specific

pathogen-free conditions. Then, the mice were separated into four groups with 6 mice of each. MLATA1-sh 4T1 cells and blank-vector 4T1 cells were pretreated with LPS (5 mg/kg) for 3 days before tumor cell inoculation, and the control group was treated with isometric phosphate-buffered saline (PBS). Approximately 1×10^5 cells suspended in 50:50 mixture of culture medium and matrigel (BD Bioscience) were injected into the coupled abdominal mammary glands of the BALB/c mice. After 3 weeks, the mice were sacrificed and the metastatic tumor

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nodules in the lungs were counted under a dissecting microscope. Before sacrifice, blood samples were collected for the IL-6 and TNF- α ELISA assay. The lungs from the different groups of mice were dissected and fixed in 4% paraformaldehyde for 48 hours, embedded in paraffin, and then were cut into slices with a thickness of 3-4 μ m and stained with H&E staining. The slices were observed under a light microscope. Western blot assays were employed to detect the expression of MMP-9 and Vimentin in the fresh lung tissues of different groups of mice. All experiments were approved by the Third Hospital of Nanchang City Experimental Animal Committee.

Statistical analysis

Statistical differences were determined by 2-tailed t-test, one-way ANOVA coupled with a Tukey's post hoc test or two-way ANOVA coupled with a Bonferroni post hoc test using the GraphPad Prism 6 program (GraphPad Software, La Jolla, CA, USA). Statistical significance was analyzed using Statistical Package for the Social Sciences (SPSS) version 19 for Windows (SPSS Inc., Chicago, IL). The Chi-square test was used to analyze the association of postoperative fever with the clinicopathological features of the tumors. Disease free survival (DFS) was calculated from the date of initial diagnosis to the date of recurrence or death. Cumulative DFS probabilities were calculated according to the Kaplan-Meier method and statistical significance was analyzed by the log-rank test. $P \leq 0.05$ represents a statistically significant difference, and all reported P values were two-sided.

Results

LncRNA MALAT1 elevated in breast cancer patients with postoperative fever and was associated with the RFS

The correlation between fever and tumor recurrence has attracted particular attention recently. The inflammatory mediators were supposed to mediate the disease relapse regulated by fever. To confirm this hypothesis, we analyzed the co-relationships between the pro-inflammatory factors and fever or tumor recurrence in 258 breast patients. The results showed that the plasma IL-6, TNF- α and MALAT1 levels in the fever group were significantly higher than

those in the non-fever group ($P = 0.0154$, <0.001 , <0.001) (**Figure 1A, 1C and 1E**).

In the univariate survival analysis, we found high expression of MALAT1 was associated poor RFS survival in 258 primary breast cancer patients (**Figure 1F**), but similar results of the IL-6 and TNF- α expression were not observed (**Figure 1B and 1D**). The short-term RFS rates for the MALAT1 high expression group and low expression group were 79.17% and 96.47%, respectively. Low MALAT1 expression predicted favorable short-term RFS in our cohort (HR = 0.0093, 95% CI: 0.0013-0.0637, $P < 0.001$) (**Figure 1F**).

LPS promoted the expression of MALAT1 and induced the invasion and migration of 4T1 cells

Lipopolysaccharide (LPS) is capable of inducing significant systemic inflammation and severe sepsis. We observed that IL-6, TNF- α and MALAT1 were elevated in the supernatants of 4T1 cells treated with LPS (**Figure 2A-C**). In addition, wound healing assay was employed to assess the 4T1 cell migration and Transwell assay was employed to assess 4T1 cell invasion. Compared with control, LPS treatment obviously increased 4T1 cell migration (**Figure 2D**) and invasion (**Figure 2E**).

MALAT1 regulated LPS-induced migration and invasion of 4T1 cells

LPS was found to be able to induce breast cancer cell invasion and metastasis via regulating NF- κ B and the Erk pathway [18]. MALAT1 may regulate the expression of inflammatory mediators in endothelial cells, including TNF- α and IL-6 [15] and induce the migration and invasion of human breast cancer cells [19]. To investigate the role of MALAT1 in LPS-induced breast cancer cell invasion and metastasis, MALAT1-shRNA lentivirus was transfected into the 4T1 cell line, and subsequently, qPCR assays revealed that MALAT1 expression was significantly reduced (**Figure 3A**). Our results showed that the LPS-induced IL-6 expression in the MALAT1-shRNA group was not different from that in the empty vector group (**Figure 3B**), but the TNF- α expression was sharply decreased ($P < 0.01$) (**Figure 3C**). Wound healing assays and Transwell assays also indicated that the cell migration and invasion induced by LPS

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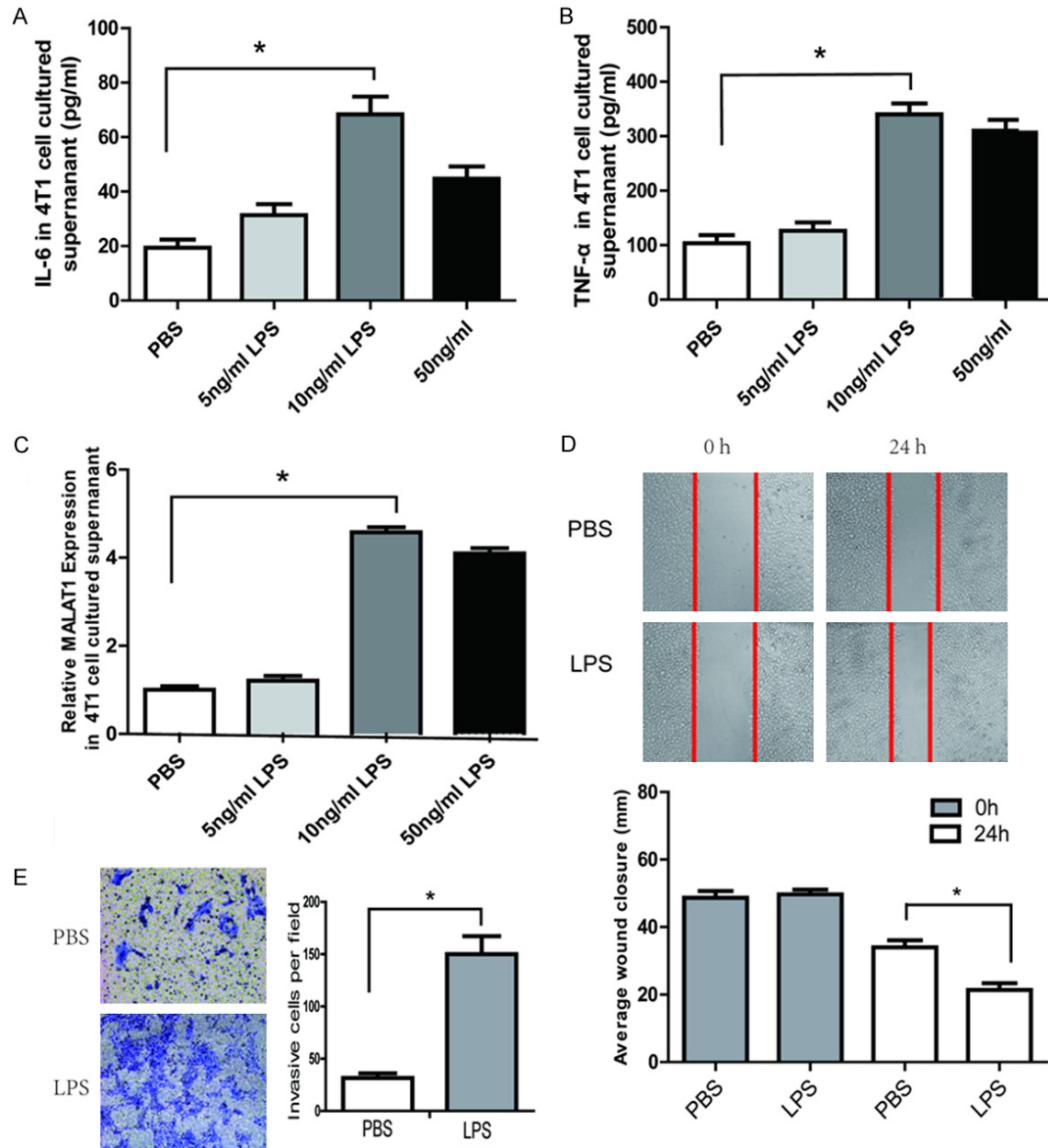


Figure 2. LPS induced IL-6, TNF- α and MALAT1 expression and promoted invasion and migration of breast cancer cells. ELISA assay of IL-6 (A) and TNF- α (B) levels in the cultured supernatant of 4T1 cells after treatment with LPS or PBS. (C) Q-PCR analysis of MALAT1 expression in 4T1 cells after treatment with LPS or PBS. (D) Effects of LPS on the migration of 4T1 cells evaluated by the scratch wound healing assay. Images were taken at 0 and 24 h after wound formation, and the wound surface areas were measured using Image J software and normalized to the initial state. Representative phase-contrast images of 4T1 cells migrating into the wounded area were shown. (E) Effects of LPS on the invasion of 4T1 cells evaluated by Transwell migration assay. Representative images of the migrating cells on the bottom of the Millicell membranes (pore size 8 μ m) was shown. All data are presented as the mean \pm SEM from three independent experiments (n = 3). *P<0.05.

decreased after knocking down MALAT1 expression (Figure 3D-G).

Moreover, qPCR and Western blot assay were applied to evaluate the mRNA and protein expression of MMP-9 and vimentin in the 4T1

cells. We found LPS promoted MMP-9 and vimentin expression, whereas these effects were decreased after MALAT1 knockdown (Figure 4A-C). These results together suggested that MALAT1 regulated the process of LPS-induced breast cancer cell migration and invasion.

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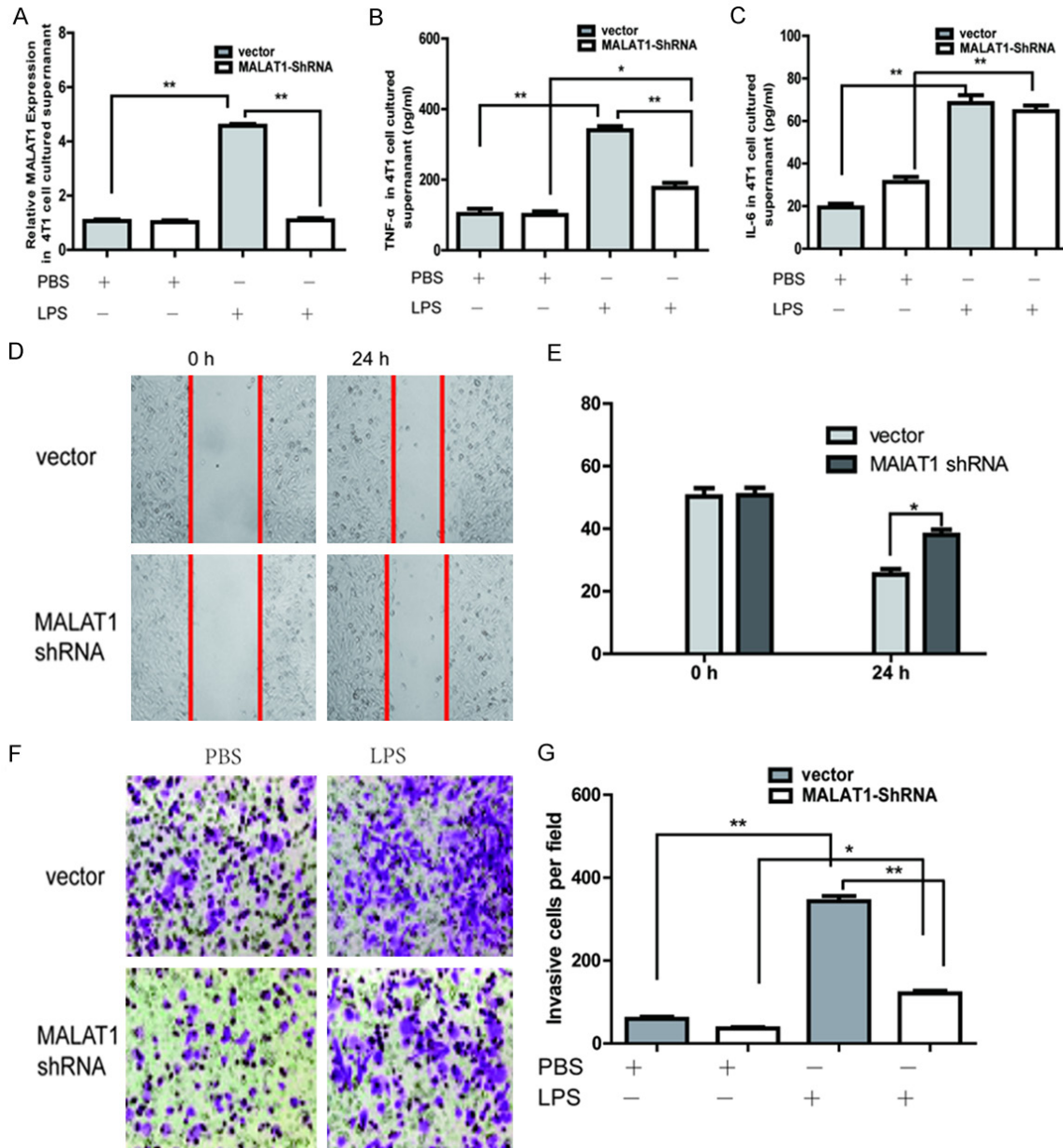


Figure 3. MALAT1 regulated the LPS-induced invasion and migration of 4T1 cells. The effect of LPS treatment on MALAT1 expression (A) and the TNF- α level (B) in the MALAT1-shRNA group was significantly reduced compared with the empty vector group. (C) The IL-6 level were almost the same in the MALAT1-shRNA group and the empty vector group after LPS treatment. (D, E) In the wound healing assay, the cell motility of the MALAT1-shRNA group induced by LPS was decreased compared with that of the empty vector group. (F, G) Transwell assay indicated that the cell migration of the MALAT1-shRNA group induced by LPS decreased compared with that of the empty vector group. All data are presented as the mean \pm SEM from three independent experiments ($n = 3$). * $P < 0.05$, ** $P < 0.01$.

LPS induced pulmonary inflammation and promoted metastasis, and this effect was inhibited after MALAT1 knockdown

As reported by Jiang *et al* [20], LPS induced systemic inflammation in BALB/c mice. In the present study, IL-6 and TNF- α levels detected by ELISA in the plasma samples of the LPS-treated

mice were found to increase compared with the PBS-treated mice (Figure 5A and 5B). Similar observation was also shown in the MALAT1 expression in the mice plasma samples (Figure 5C). There was a significant increase in edema and the lung wet/dry ratio in LPS-treated mice compared with PBS-treated mice (Figure 5D and 5E). In the H&E staining, all the

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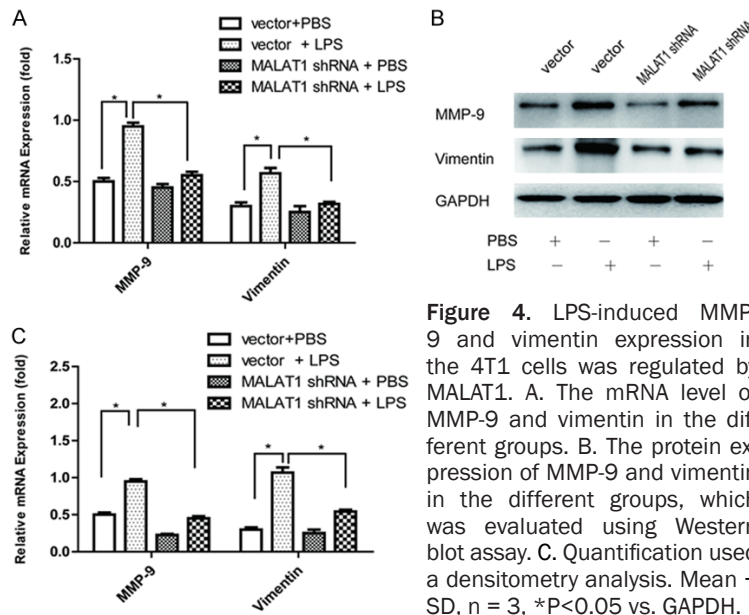


Figure 4. LPS-induced MMP-9 and vimentin expression in the 4T1 cells was regulated by MALAT1. A. The mRNA level of MMP-9 and vimentin in the different groups. B. The protein expression of MMP-9 and vimentin in the different groups, which was evaluated using Western blot assay. C. Quantification used a densitometry analysis. Mean + SD, n = 3, *P<0.05 vs. GAPDH.

lungs in LPS-treated mice were found inflammatory cell infiltration characterized by prominent neutrophilic and granulocytic cells compared with PBS-treated mice. Interestingly, the LPS-induced inflammation response including IL-6, TNF- α and MALAT1 expression in the plasma samples as well as the pulmonary inflammation in the H&E staining in the MALAT1-shRNA xenograft mouse were inhibited when compared with that in the empty-vector counterparts (**Figure 5A-E**).

In addition, the number and size of the lung metastases significantly increased in LPS-treated mice than in PBS-treated mice (**Figure 5D** and **5F**). The different expression of MMP-9 and vimentin in the fresh lung tissues between LPS-treated mice and PBS-treated mice further support the hypothesis that LPS promoted the breast cancer lung metastases in vivo (**Figure 5G** and **5H**). More specifically, the LPS-induced lung metastasis and EMT-related protein (MMP-9 and Vimentin) expression were inhibited after MALAT1 knocked down. Thus, MALAT1 is a key regulator of LPS-induced pulmonary inflammation and metastasis in xenografted model.

Discussion

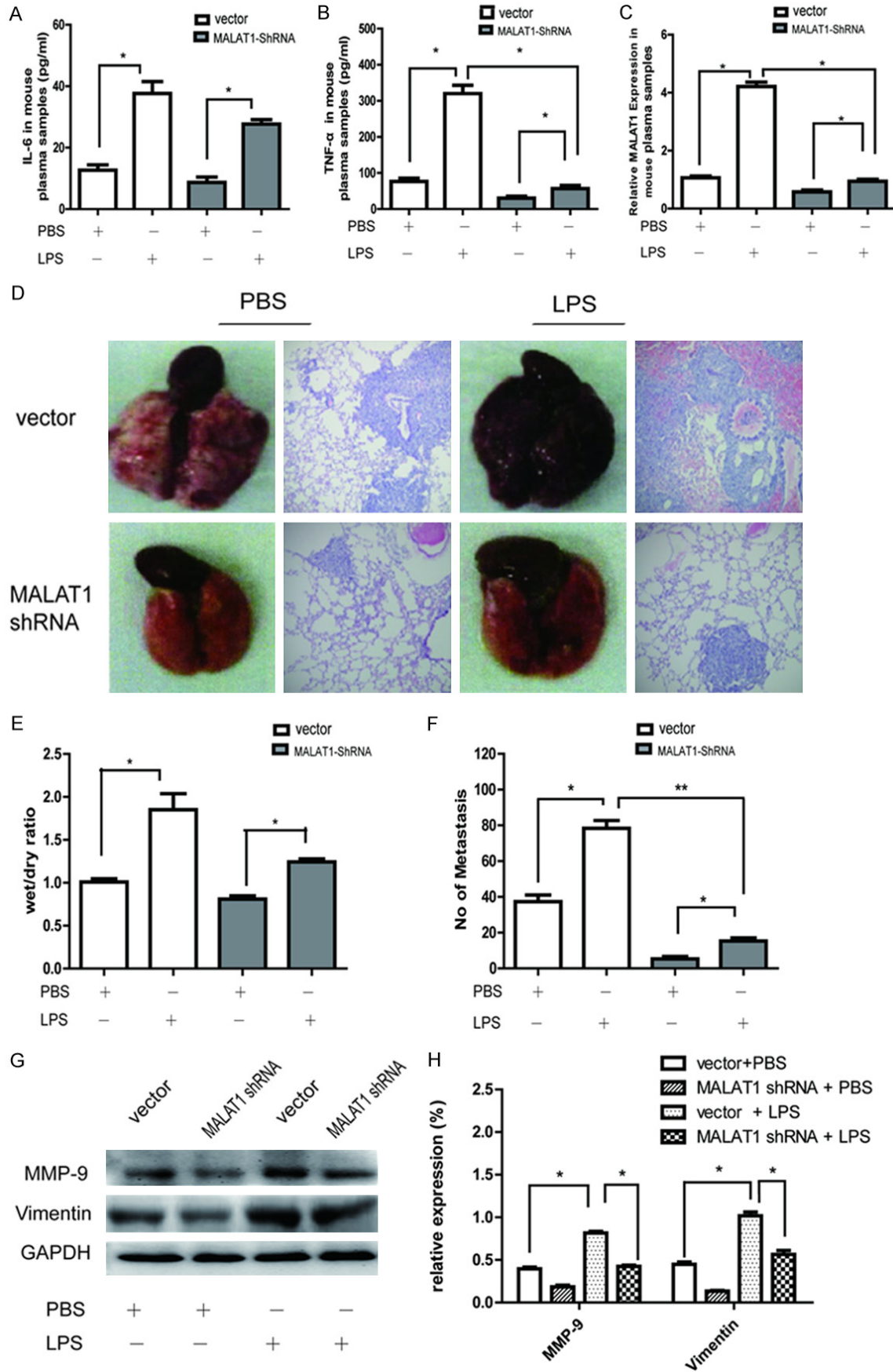
Fever is prevalent in many postoperative patients, with an incidence ranging from 13 to 39%. Infectious factors should be considered for fever presenting later than 48 hours after

surgery, whereas early postoperative fever is usually deemed as the host response to surgery [21, 22]. Interestingly, some retrospective studies proposed that postoperative fever might also serve as a rough indicator for the poor prognosis of breast cancer patients [9, 10]. To investigate the association between postoperative fever and short-term relapse, 258 newly admitted breast cancer patients who underwent surgical treatment in the Third hospital of Nanchang City were enrolled in this study during June 15, 2014 to December 15, 2015. Our results showed that the short-term relapse-free survival rate of

the fever group and the non-fever group were 90.2% and 97.5%, respectively, and the difference between them was statistical significant (P = 0.0149), which was shown in [Supplementary Data](#).

It is well known that postoperative fever refers to a rise in the core temperature, which is most commonly caused by inflammatory changes due to the release of pyrogenic cytokines, such as IL-6 and TNF- α [23]. These cytokines may be induced by infection, injury, surgery, general anesthesia trauma or be secreted by the tumor itself. Previous studies have elaborated on the independent negative impact on prognosis of high levels of IL-6 or TNF- α in the serum of patients with breast cancer [24]. Our study also found that the expression of plasma IL-6 or TNF- α in postoperative fever patients with breast cancer increased, but these high expression of the two pre-inflammatory factors described above was not associated with the adverse prognosis of breast cancer patients. In other words, IL-6 or TNF- α were not the root of the postoperative fever that was associated with the poor prognosis of breast cancer patients, and maybe some other cytokines were involved in this process. Recently, the long non-coding RNA MALAT1 was also considered a pro-inflammatory factor, which regulated the lipopolysaccharide-induced inflammatory response [25] and EMT process of breast cancer cells [16, 26]. Interestingly, our study indicated that

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Figure 5. LPS induced pulmonary inflammation and promoted metastasis, while knocking down MALAT1 reduced the effect in vivo. The xenograft mice were divided into four groups including PBS + vector group, PBS + MALAT1-shRNA group, LPS + vector group and LPS + MALAT1-shRNA group. (A, B) ELISA assay of the plasma IL-6 (A) and TNF- α (B) levels in the different xenograft mice. (C) Q-PCR analysis of MALAT1 expression in the plasma samples of the different xenograft mice. lung morphology and H&E staining of the lung in different xenograft mice. (D) The edema and lung wet/dry ratio, inflammatory cellular infiltrates and the number of the lung metastasis were facilitated by LPS, but the inducing effect was decreased in the MALAT1-shRNA group compared to the empty vector group. (E) The quantitative analysis of edema and the lung wet/dry ratio in the different groups. (F) The quantitative analysis of the lung metastasis in the different groups. All data are presented as the mean \pm SEM (n = 3). *P<0.05, **P<0.01 compared with control (empty vector). (G) The difference in the expression of MMP-9 and vimentin in the fresh lung tissues of different groups. (H) Quantification used a densitometry analysis. Mean + SD, n = 3, *P<0.05 vs. GAPDH.

there was a difference in the expression of plasma MALAT1 between the fever group and the non-Fever group. High MALAT1 expression predicted the adverse short-term DFS of these patients.

Lipopolysaccharide (LPS), which is in the outer cell membrane of Gram-negative bacteria, can induce significant systemic inflammation and several pro-inflammatory cytokines secretion, including IL-6, tumor necrosis alpha (TNF- α) etc. [27]. Our study found that the expression of IL-6 and TNF- α in the supernatants of 4T1 cells was elevated in the LPS-treated group compared to the control group, which was detected by ELISA assay. Similarly, our qRT-PCR assay indicated that the expression of MALAT1 was also increased after LPS treatment. Thus, LPS was believed to promote the expression of IL-6, TNF- α and MALAT1 in the 4T1 cells. However, after knocking down the expression of MALAT1 in 4T1 cells, the TNF- α expression was dramatically decreased while the IL-6 expression remain unchanged. Recently, *Zhang et al* reported that IL-6-induced lncRNA MALAT1 enhances TNF- α expression in LPS-induced septic cardiomyocytes [28]. *Puthanveetil et al* also found that the lncRNA MALAT1 regulates the glucose-induced up-regulation of the inflammatory mediators IL-6 and TNF- α through the activation of SAA3 [15]. Our research herein may provide a new mechanism by which lncRNA MALAT1 participated in the regulation of the LPS-induced inflammatory response in breast cancer cells.

LPS is also the primary ligand for TLR4, which can induce the inflammation-associated invasion and metastasis of breast cancer cells [29, 30]. Our data indicated that treating 4T1 cells with LPS could increase cell migration and invasion, which can be blocked by MALAT1 knock-down. Recent report demonstrated that the long noncoding RNA MALAT1 regulates the LPS-induced inflammatory response through

its interaction with NF- κ B [31]. It has been shown that NF- κ B, a key protein regulating the immune and inflammatory process, also plays an important role in regulating the EMT process [32]. Notably, matrix metalloproteinase 9 (MMP9), which is directly regulated by NF- κ B, is a proteolytic enzyme which particularly plays a role in the early steps of cancer cell invasion [33, 34]. Moreover, NF- κ B is one of the transcription factors that binds to the promoter of vimentin which is overexpressed during the epithelial-mesenchymal transition (EMT) process [35, 36]. Our data also indicated that LPS promoted MMP-9 and vimentin expression, whereas these effects were inhibited after knocking down MALAT1 expression. Therefore, MALAT1 is believed to induce the migration and invasion of human breast cancer cells [19], and may also contribute to the postoperative fever induced recurrence in patients.

In conclusion, early postoperative fever was an independent risk factor for the prognosis of breast cancer patients. Specific inflammatory cytokines released from the breast cancer patients induced a systemic inflammatory response and postoperative fever. As an important pre-inflammatory factor, MALAT1 is involved in the regulation of the systemic inflammatory response and promotes the invasion and metastasis of breast cancer. And MALAT1 is believed to engage in the recurrence, metastasis and postoperative fever in breast cancer patients.

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Disclosure of conflict of interest

None.

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Supplementary Data

Postoperative fever and the short-term recurrence of breast cancer patients. All the enrolled patients completed the follow-up, with a median follow-up of 23 months (range 3-36 months). During the follow-up period, 11 relapse events were observed in 258 breast cancer patients, including 1 case with supra-clavicular lymph node metastasis, 2 cases with bone metastasis, 1 case with liver metastasis, 3 cases with pulmonary pleural metastasis, and 4 cases with multiple organ metastasis. Before the end of follow-up, 3 patients died due to relapse events. When compared with patients without fever after surgery (Non-fever group), those who had postoperative fever (Fever group) experienced more relapse events. Relapse-free survival of the two groups was 97.5% to 90.2% (Figure S1A), respectively. In the univariate survival analysis, we found significantly different PFS between the two groups (Log-rank test, $P = 0.0149$, Figure S1B). In the Cox proportional hazards regression analysis, we found that tumor size ($P = 0.015$; RR = 4.42; 95% CI 1.34-14.58) and fever ($P = 0.043$; RR = 3.43; 95% CI 1.04-11.31) were independent prognostic factors for PFS (Table S3).

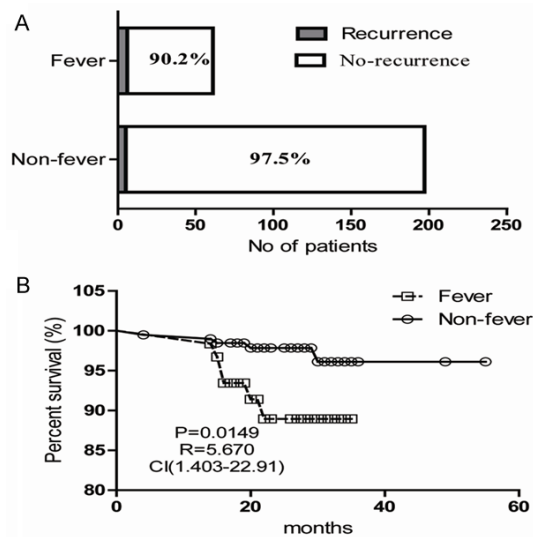


Figure S1. Postoperative fever may be an indicator of the short-term recurrence of breast cancer patients. A. Relapse-free survival of the fever group and non-fever group was 97.5% and 90.2%, respectively. B. Kaplan-Meier curve analysis indicated that patients with postoperative fever had poor prognosis compared with patients without postoperative fever (Log-rank test $P = 0.0149$).

Table S1. The nucleotide sequences of the targeting MALAT1 and Negative control shScramble

Malat1 siRNA-1	Sense	CACAGGGAAAGCGAGTGGTTGGTAA
	Antisense	TTACCAACCACTCGCTTTCCTGTG
Malat1 siRNA-2	Sense	GAGGUGUAAAGGGAUUUUAUTT
	Antisense	AUAAAUCCCUUUACACCUCTT
Malat1 siRNA-3	Sense	GGAAGATAGAAACAAGATATATCTTGTTCCTATCTCC
	Antisense	GGAAGATAGAAACAAGATATATCTTGTTCCTATCTCC
NC shScramble	Sense	UUCUCCGAACGUGUCACGUTT
	Antisense	ACGUGACACGUUCGGAGATT

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Table S2. The primers for MMP3 and vimentin

Gene	Foward primer (5'→3')	Reverse primer (5'→3')
MMP9	CAGCCCCTGCTCCTGGCTCTCCTG	ACTCGTCGTCGTCGAAATGGGCAT
Vimentin	GACAATGCGTCTCTGGCAGTCTT	TCCTCCGCCTCCTGCAGGTTCTT
β-actin	TCATGAAGTGTGACGTGGACATC	CAGGAGGAGCAATGATCTTGATCT

Table S3. Multivariate Cox regression analysis of prognostic factors for DFS in 258 primary breast cancer patients

Variable	RR	95% CI of RR	P value
Age (≤50 vs. >50)	0.567	0.157~2.055	0.388
Tumor-size (≥5 cm vs. <5 cm)	4.420	1.340~14.577	0.015
Lymph node status (positive vs. Negative)	2.197	0.511~9.441	0.290
ER (≥1% vs. <1%)	0.394	0.040~3.841	0.423
PR (≥1% vs. <1%)	0.683	0.062~7.503	0.756
HER2 (positive vs. Negative)*	2.576	0.684~9.702	0.162
Ki-67 (<20% vs. ≥20%)	0.316	0.074~1.345	0.119
Fever (≥38 °C vs. <38 °C)	3.430	1.040~11.313	0.043

*HER2-positive cancers were defined by either strong membrane staining (3+) observed by IHC or amplification of HER2 confirmed by fluorescence in situ hybridization when immunohisto-chemistry detected moderate (2+) membrane staining.