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Original Article

Elevated expression of LIMK2 is an independent prognostic indicator in breast cancer

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Abstract: Background: LIM domain kinase 2 (LIMK2) plays an important role in cell proliferation, adhesion, migration, differentiation and inflammation. Abnormal expression of LIMK2 is implicated in various malignancies, but little is known about its expression and prognostic value in breast cancer. Methods: Specimens were collected from 212 patients for the analysis of LIMK2 expression by immunohistochemistry. Kaplan-Meier analysis and Cox regression analysis were performed to evaluate the prognostic significance of LIMK2 in breast cancer. The same analyses were conducted using METABRIC and the Kaplan Meier plotter databases to validate the expression pattern and prognostic value of LIMK2. Results: LIMK2 was located in the cytoplasm, and the positive rate of LIMK2 expression in the breast cancer tissues was significantly higher than that in normal breast tissues ($P<0.0001$). Elevated LIMK2 expression correlated with large tumor size and high histological grade ($P<0.05$ for each). Kaplan-Meier analysis showed elevated expression of LIMK2 predicted worse disease-free survival (DFS) (HR: 3.295, 95% CI: 2.251-7.156, $P<0.0001$) and overall survival (OS) (HR: 6.251, 95% CI: 3.874-16.260, $P<0.0001$). Multivariable Cox regression analysis indicated elevated expression of LIMK2 was an independent prognostic factor for both DFS and OS (for DFS, HR: 2.707, 95% CI: 1.546-4.740, $P=0.0005$; for OS, HR: 5.241, 95% CI: 2.436-11.277, $P<0.0001$). The expression pattern and prognostic significance of LIMK2 in breast cancer patients were validated by bioinformatics analysis of public databases. Conclusion: In conclusion, our results suggest that LIMK2 is an independent poor prognostic factor for breast cancer patients, and LIMK2 might play an important role in the progression of breast cancer.

Keywords: LIMK2, breast cancer, expression, prognosis

Introduction

Invasion and metastasis are the main causes of death in breast cancer. Mounting evidence suggested that LIM domain kinases (LIMK), key regulators of the actin cytoskeleton, was prominently associated with tumorcell invasion and metastasis [1, 2]. Typical structure of LIMKs is comprised with two LIM domains at the N-terminus, a PDZ domain connected to proline/serine-rich regions and a C-terminal kinase domain [3, 4]. LIMK family has two members, LIMK-1 and LIMK-2 [4]. Though they share about 50% of overall identical sequence, different expression profile, subcellular and organic location and function were observed between them [5-7].

LIMKs are play an important role in cell proliferation, adhesion, migration, differentiation and inflammation [7-13]. The overexpression of LIMK1 was detected in melanoma, prostate and breast cancer [14-17]. Recently, LIMK2 was reported to be required for the formation of invadopodia, matrix degradation and invasion as well as migration of breast cancer cells [13], which suggested that LIMK2 might be involved in the progression of breast cancer; however, the expression pattern of LIMK2 in breast cancer and its correlations with prognosis of breast patients is poorly understood.

To investigate the prognostic value of LIMK2 in breast cancer patients, specimens from 212 patients were collected for the analysis of LIMK2 expression by immunohistochemistry (IHC). The relationship between LIMK2 expression and clinicopathological parameters of breast cancer was evaluated. Meanwhile, the prognostic roles of LIMK2 in breast cancer were analyzed with Cox regression and Kaplan-Meier analysis. To validate the expression pattern and prognostic value of LIMK2, the same analyses were conducted using METABRIC and the Kaplan Meier plotter databases. Our data implicated that elevated LIMK2 expression is an independent poor prognostic factor for breast cancer patients.

Table 1. Clinical and pathological characteristics of all 212 breast cancer patients

Characteristics	Number of patients (%)
Total	212
Age (years)	
<50	99 (46.70%)
≥50	113 (53.30%)
Location	
Left	122 (57.55%)
Right	90 (42.45%)
pT Stage	
0	3 (1.42%)
1	65 (30.66%)
2	121 (57.08%)
3	8 (3.77%)
4	15 (7.08%)
pN stage	
0	99 (46.70%)
1	84 (39.62%)
2	10 (4.72%)
3	19 (8.96%)
M stage	
0	203 (95.75%)
1	9 (4.25%)
TNM stage	
0	3 (1.42%)
1	34 (16.04%)
2	125 (58.96%)
3	41 (19.34%)
4	9 (4.25%)
Histological type	
Infiltrating ductal carcinoma	179 (84.43%)
Infiltrative lobular carcinoma	13 (6.13%)
Ductal carcinoma in situ	10 (4.72%)
Others	11 (5.19%)
Histological grade	
1	43 (20.28%)
2	144 (67.92%)
3	25 (11.79%)
ER	
Positive	140 (66.04%)
Negative	72 (33.96%)
PR	
Positive	109 (51.42%)
Negative	103 (48.58%)
HER2	
Positive	33 (15.57%)
Negative	179 (84.43%)

Materials and methods

Patients and tissue samples

The present study was conducted with the approval of the Ethical and Scientific Committees of Southwest Hospital, Third Military Medical University (Chongqing, China). Patients were informed that the specimens would be used for scientific research, and their privacy would be maintained.

A number of 212 breast cancer patients who treated with surgery at the Southwest Hospital between February 2006 and June 2009 were identified. No treatment were received before surgery. The patients' age ranged between 25 and 79 years with a mean age of 51.4 years. The mean follow-up time is 66 months. Patients' characteristics are shown in **Table 1**. Two experienced pathologists observed the hematoxylin and eosin-stained slides of the different biopsies according to the World Health Organization classification guidelines. Additionally, 17 samples of normal breast tissue were used as normal controls.

Immunohistochemical staining

The ChemMate™ Envision™ Detection Kit (Dako, Carpinteria, CA, USA) was used for IHC according to the manufacturer's instructions. Briefly, the samples were fixed by 10% formaldehyde and embedded by paraffin, and then 4-um section-cut specimens was performed. All of the sections were dewaxing and hydration with dimethylbenzene and a gradient concentration of alcohol. Deionized water and phosphate-buffered saline washed the sections, and then an antigen retrieval process was performed at high temperature and high pressure with citrate buffer (pH 6.0). The endogenous peroxidase was locking with 0.3% (v/v) H_2O_2 solution. For reducing nonspecific reaction, the sections were then incubated with goat serum for 60 min Then, the sections were

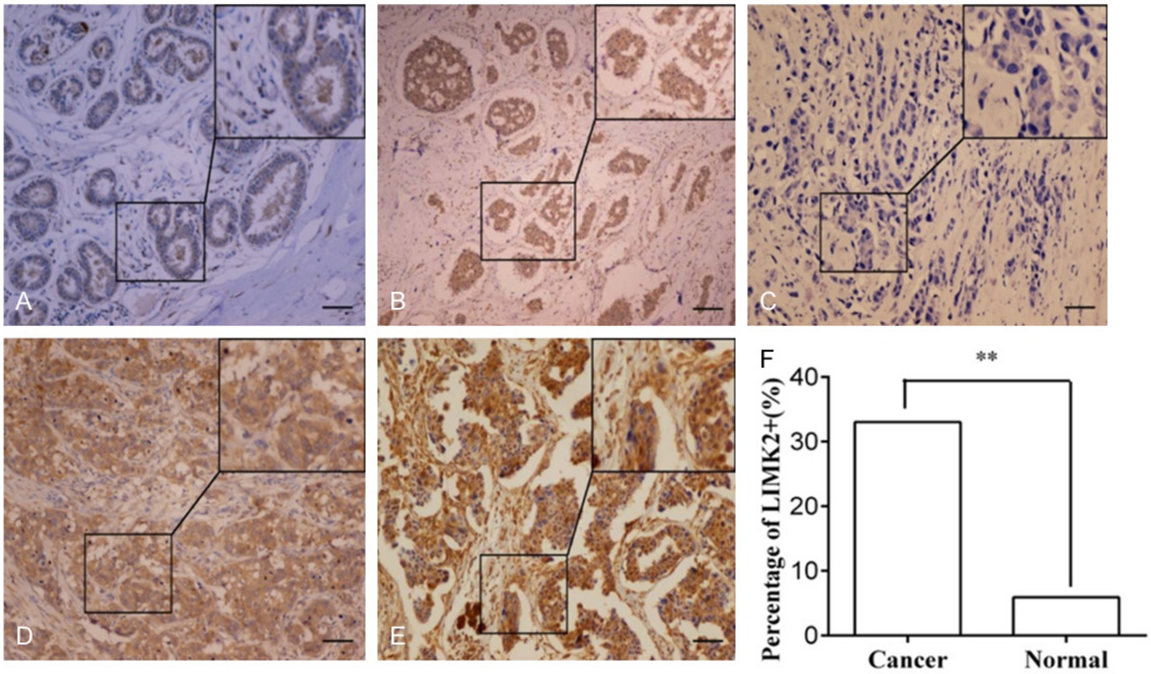

Figure 1. LIMK2 expression in breast cancer and adjacent normal specimens. (A and B) IHC staining performed for LIMK2 in normal breast tissues, and LIMK2 negative (A) and positive (B) images were shown. Bar, 50 um. (C-E) Weak (C), moderate (D) and strong (E) staining of LIMK2 in breast cancer tissues. Positively reactive substance of LIMK2 was mainly localized in the cytoplasm. Bar, 50 um. (F) The positive rate of LIMK2 expression in breast cancer tissues and normal breast tissues (33.02% vs. 5.882%, $^{**}P<0.0001$).

incubated with LIMK2 antibody (1:50 dilutions; HPA008183, Sigma-Aldrich, St. Louis, MO, USA), overnight at 4°C. After thawing, the sections were rinsed five times with PBS, then incubated with ChemMate™ Envision™/HRP, rabbit/mouse reagent as a secondary antibody. Subsequently, the sections were treated using ChemMate™ DAB+ Chromogen (Dako, Carpinteria, CA, USA) and counterstained with hematoxylin. After dehydration and transparency with a gradient concentration of alcohol and dimethylbenzene, all of the specimens were finally fixed using neutral balsam.

Evaluation of staining

Slides were reviewed under a light microscope three time by two observers, who did not know the identity of the specimens between evaluations. Brown-yellow or brown granular deposits at the corresponding antibody expression sites indicated a positive expression result. LIMK2 is expressed in the cytoplasm, and rarely positive expression in the nucleus. The percentage of positive cells was evaluated and scored according to the following categories: 0, less than 5%; 1, 5-25%; 2, 25-50%; 3, 50-75%; 4, greater than 75%. The intensity of staining cells was recorded in the following categories: 0: no staining; 1: weak staining; 2: strong staining. The two scores were summed to obtain an immunoreactivity score (IRS) value ranging from 0 to 6. To evaluate the association of LIMK2 expression with clinical and pathological parameters, the patients were then grouped into two categories based on IRS values: low-expression (IRS 0-5) and high-expression (IRS 6).

Bioinformatics analysis

To validate the expression pattern and prognostic significance of LIMK2, retrospective analyses were conducted based on METABRIC database, which includes genomic profiles of 2,509 breast cancer patients through OASIS platform [18], and Kaplan Meier plotter database [19], which includes information on survival of 5,143 breast cancer patients.

Statistical analysis

Statistical calculations were performed using SAS software (version 9.3; SAS Institute, Cary,

Table 2. Relationship between clinical and pathological characteristics and LIMK2 expression

Characteristics	LIMK2 expression (N, %)		*P*
	Negative	Positive	
Total	142 (66.98%)	70 (33.02%)	
Age (years)			
<50	73 (73.74%)	26 (26.26%)	0.0502
≥50	69 (61.06%)	44 (38.94%)	
Location			
Left	82 (67.21%)	40 (32.79%)	0.9334
Right	60 (66.67%)	30 (33.33%)	
T stage			
0+1+2	53 (77.94%)	15 (22.06%)	0.0197
3+4	89 (61.81%)	55 (38.19%)	
N stage			
0	71 (71.72%)	28 (28.28%)	0.1699
1+2+3	71 (62.83%)	42 (37.17%)	
TNM stage			
0+1+2	113 (69.75%)	49 (30.25%)	0.1224
3+4	29 (58.00%)	21 (42.00%)	
Histological grade			
1	36 (83.72%)	7 (16.28%)	0.0089
2+3	106 (62.72%)	63 (37.28%)	
ER status			
Positive	92 (65.71%)	48 (34.29%)	0.5844
Negative	50 (69.44%)	22 (30.56%)	
PR status			
Positive	75 (68.81%)	34 (31.19%)	0.5608
Negative	67 (65.05%)	36 (34.95%)	
HER2 status			
Positive	23 (69.70%)	10 (30.30%)	0.7181
Negative	119 (66.48%)	60 (33.52%)	

NC, USA). GraphPad Prism (version 6.0; GraphPad Software Inc., La Jolla, CA, USA) was used to analyze the expression of LIMK2 between breast cancer tissues and normal tissues. The expression of LIMK2 with clinicopathological parameters was analyzed by Pearson's Chi-squared tests and Fisher's exact test. Disease-free survival (DFS) was defined as the time from surgery to recurrence of breast cancer (in the breast or chest wall or at nodal or metastatic sites). Overall survival (OS) was defined as the time from surgery to any cause of death. Survival analyses were performed using the Kaplan-Meier method, and differences between groups were assessed using the log-rank test. Univariate analysis comparisons and multivariate survival comparisons were performed using Cox proportional hazard regression models. The estimated relative risks of deaths or relapse were expressed as adjusted hazard ratios (HRs) and corresponding 95% confidence intervals (CIs). A *P* value less than 0.05 was considered statistically significant.

Results

LIMK2 was highly expressed in breast cancer and mainly located in the cytoplasm

In normal breast tissues, the cells showed no or weak staining of LIMK2 (**Figure 1A** and **1B**). In the breast cancer tissues, the positively reactive substance of LIMK2 was mainly located in the cytoplasm, and showed scarcely positive expression in the nucleus (**Figure 1C-E**). The positive rate of LIMK2 expression in the cytoplasm was notably higher in breast cancer tissues (33.02%, 70/212) than in normal breast tissues (5.882%, 1/17) (*P*<0.0001, **Figure 1F**).

The positive rate of LIMK2 was higher in breast cancers patients with tumor larger than 5 cm

The relationships between LIMK2 and clinicopathological parameters of breast cancer patients were analyzed (**Table 2**). The positive rate of LIMK2 was higher in breast cancers patients with larger diameter (>5 cm) than in cases with smaller size (<5 cm) (*P*=0.0197). The positive rate of LIMK2 expression was also higher in breast cancers with high histological levels than in cases with low histological levels (*P*= 0.0089). There were no associations between LIMK2 expression and age, location, N stage, TNM stage, ER, PR and HER2 expression (*P*>0.05 for each).

High expression of LIMK2 was an independent prognostic factor for both DFS and OS

Breast cancer patients with high LIMK2 expression had significantly lower DFS than those with low LIMK2 expression (HR: 3.295, 95% CI: 2.251-7.156, *P*<0.0001; **Figure 2A**). Breast

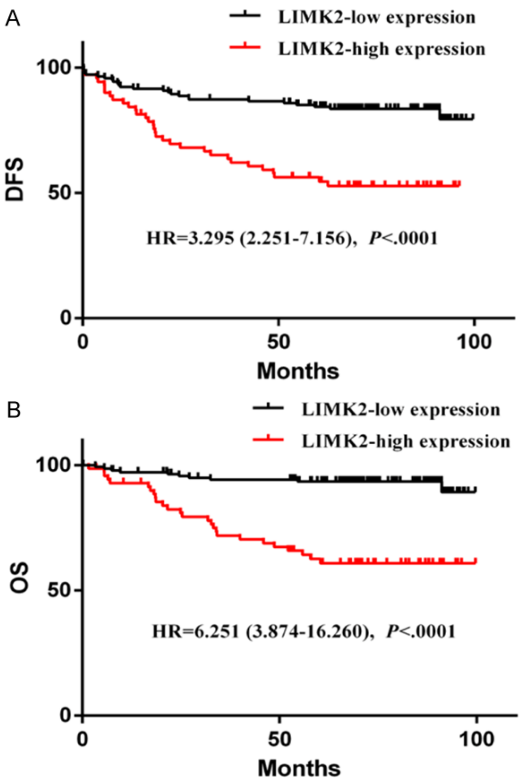

Figure 2. Prognostic value of LIMK2 expression in breast cancer patients. Kaplan-Meier analysis of disease-free survival (A) and overall survival (B) for breast cancer patients with high and low LIMK2 expressing tumors.

cancer patients with high LIMK2 expression had significantly lower OS than those with low LIMK2 expression (HR: 6.251, 95% CI: 3.874-16.260, *P*<0.0001; **Figure 2B**). These data suggest that LIMK2 maybe a independent prognostic factor in breast cancer.

Multivariate analyses were performed using the Cox proportional hazards model. We found that tumor size, TNM stage and HER2 expression were proved to be independent prognostic factors for both DFS and OS. Most importantly, elevated LIMK2 expression emerged as an independent prognostic factor for both DFS and OS (for DFS, HR: 2.707, 95% CI: 1.546-4.740, *P*=0.0005; for OS, HR: 5.241, 95% CI: 2.436-11.277, *P*<0.0001) (**Table 3**).

High expression of LIMK2 mRNA indicated poor outcomes of breast cancer patients

To confirm the expression pattern of LIMK2 in breast cancer, we queried the METABRIC database, in which 160 normal breast tissues and 1161 breast cancer tissues were interrogated to evaluate the mRNA expression level of LIMK2. Expression of LIMK2 was significantly increased in breast cancer in comparison with normal tissue (**Figure 3A**, *P*<0.0001). To confirm the prognostic value of LIMK2 in breast cancer, the Kaplan Meier plotter, which could assess the effect of 54,675 genes on survival using 5,143 breast cancer patients, were explored. Elevated mRNA expression of LIMK2 predicted worse relapse-free survival (RFS) (HR: 1.13, 95% CI: 1.01-1.27, *P*=0.038; **Figure 3B**) and OS (HR: 1.52, 95% CI: 1.20-1.92, *P*< 0.001; **Figure 3C**).

Discussion

To our knowledge, this is the first study to explore the expression pattern of LIMK2 in breast cancers and correlate its expression level with clinicopathological characteristics and prognosis of breast cancer patients. Our results indicated elevated expression of LIMK2 was associated with large tumor size and high histological grade, and most importantly, it was an independent poor prognostic factor for breast cancer patients.

LIMK2 belongs to LIMK family, which includes LIMK1 and LIMK2. LIMK1 gene locates on human chromosome 7q11, which included 16 exons; whereas, LIMK2 gene locates on human chromosome 22q12.2, which included 19 exons. Both of them have a PDZ domain structure. Two nuclear signal output regions with leucine were found in this domain, which affected the LIMK nucleoplasm shuttle [1, 4, 20, 21]. LIMK1 was overexpressed in breast cancer and its important role in breast cancer tumor growth, angiogenesis and invasion had been demonstrated by numerous studies [16, 22-24]. In the meanwhile, LIMK2 could also contribute to chemotherapy resistance in neuroblastoma cell lines [10, 12] and p53-mediated survival of cancer cells following DNA damage [25]. Moreover, T56-LIMKi, an inhibitor of LIMK2, could effectively inhibit growth of pancreatic cancer [26]. These results indicated LIMK2 might play an important role in cancer cell progression, but little was known about the expression pattern and biological effects of LIMK2 in breast cancer.

Previous studied suggested that LIMK2 served as substrate and key oncogenic effecter of

Table 3. Multivariate analyses of disease-free survival and overall survival for positive (+) or netative (-) expression of LIMK2

	DFS			OS		
	HR	95% CI	*P*	HR	95% CI	*P*
Age	0.988	0.963-1.014	0.3803	1.002	0.972-1.033	0.8919
Location (right vs. left)	0.689	0.396-1.198	0.1866	0.904	0.452-1.809	0.7761
T (T3+T4 vs. T1+T2)^{a}	3.049	1.249-7.441	0.0143	3.426	0.991-11.84	0.0516
N (N1-3 vs. N0)^{b}	1.513	0.748-3.060	0.2497	1.332	0.543-3.271	0.5313
TNM (III+IV vs. I+II)^{c}	2.975	1.594-5.552	0.0006	2.938	1.329-6.492	0.0077
Grade (II+III vs. I)^{d}	0.617	0.245-1.552	0.3046	0.769	0.205-2.892	0.6981
ER (+ vs. -)^{e}	0.973	0.509-1.861	0.9346	0.83	0.347-1.987	0.6763
PR (+ vs. -)^{f}	0.659	0.346-1.258	0.2061	0.745	0.318-1.743	0.4971
HER2 (+ vs. -)^{g}	2.219	1.139-4.324	0.0192	2.721	1.227-6.030	0.0137
LIMK2 (+ vs. -)^{h}	2.707	1.546-4.740	0.0005	5.241	2.436-11.277	<.0001

DFS, disease-free survival; OS, overall survival. a: T1+T2 was reference group; b: N0 was reference group; c: I+II was reference group; d: I was reference group; e: ER- was reference group; f: PR- was reference group; g: HER2- was reference group; h: LIMK2- was reference group.

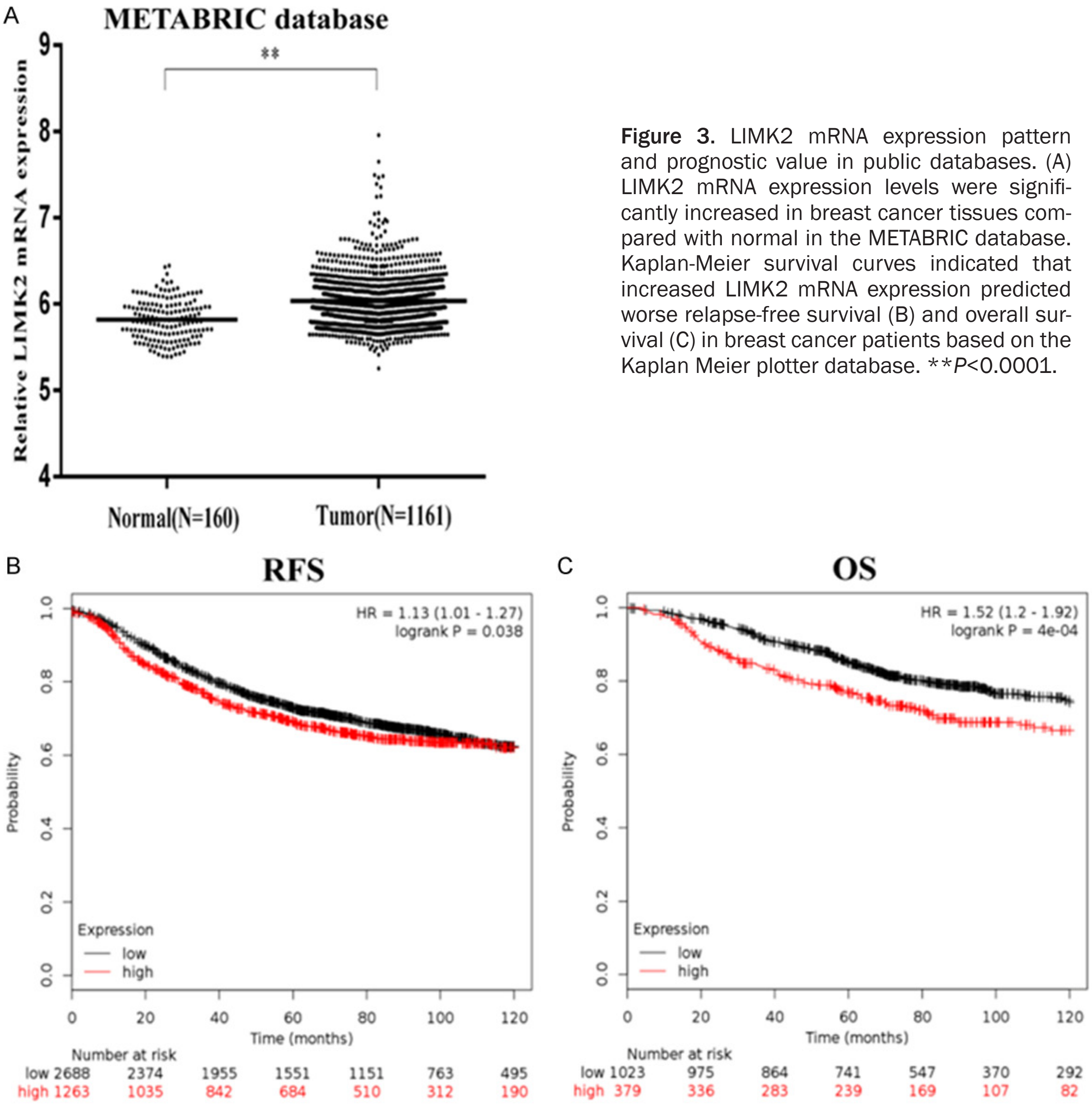

Figure 3. LIMK2 mRNA expression pattern and prognostic value in public databases. (A) LIMK2 mRNA expression levels were significantly increased in breast cancer tissues compared with normal in the METABRIC database. Kaplan-Meier survival curves indicated that increased LIMK2 mRNA expression predicted worse relapse-free survival (B) and overall survival (C) in breast cancer patients based on the Kaplan Meier plotter database. ***P*<0.0001.

Aurora A in breast cancer cells. Moreover, Aurora A and LIMK2 could be engaged in a positive-feedback loop, promoting Aurora-A-mediated oncogenic pathways [11]. In addition, recent report found LIMK2 was involved in the formation of invadopodia, matrix degradation and invasive migration in breast cancer cells. Nevertheless, to the best of our knowledge, the expression pattern and prognostic value of LIMK2 in breast cancer was still not clarified. To certify the biological role of LIMK2 in breast cancer, we collected specimens from 212 patients and correlated the LIMK2 expression with clinicopathological factors and survival of breast cancer patients using Kaplan-Meier analysis and Cox regression analysis. Our results suggested that LIMK2 was an independent poor prognostic factor for breast cancer patients, which was subsequently confirmed by bioinformatics analysis METABRIC and the Kaplan Meier plotter databases.

In conclusion, our data implied that elevated LIMK2 expression was an independent poor prognostic factor for breast cancer patients, which suggested LIMK2 might have a critical role in the progression of breast cancer. Detection of LIMK2 could provide some suggestions for diagnosis, prognostication and personalized treatment for breast cancer.

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Disclosure of conflict of interest

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