Enhanced Expression of LKB1 in Breast Cancer Cells Attenuates Angiogenesis, Invasion, and Metastatic Potential

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Abstract

***LKB1* (also known as *STK11*) is a recently identified tumor suppressor gene whose mutation can lead to Peutz-Jeghers syndrome, which is characterized by gastrointestinal polyps and cancers of different organ systems. Approximately 30% of sporadic breast cancer samples express low levels of LKB1. This suggests that the *LKB1* gene may be related to the tumorigenesis of breast cancer. We reintroduced LKB1 into MDA-MB-435 breast cancer cells that lack the *LKB1* gene to investigate how overexpression of LKB1 affects tumor invasiveness and metastasis. Overexpression of the LKB1 protein in breast cancer cells resulted in significant inhibition of *in vitro* invasion. *In vivo*, LKB1 expression reduced tumor growth in the mammary fat pad, microvessel density, and lung metastasis. LKB1 overexpression was associated with down-regulation of matrix metalloproteinase-2, matrix metalloproteinase-9, vascular endothelial growth factor, and basic fibroblast growth factor mRNA and protein levels. Overexpression of the LKB1 protein in human breast cancer is significantly associated with a decrease in microvessel density. Our results indicate that LKB1 plays a negative regulatory role in human breast cancer, a finding that may lead to a new therapeutic strategy.**

(Mol Cancer Res 2006;4(11):843–9)

Introduction

The *LKB1/STK11* gene (locus on 19p13.3) encodes a serine/threonine protein kinase and is deficient in the majority of patients with Peutz-Jeghers syndrome (PJS; ref. 1). PJS is an autosomal-dominant inherited disorder, which is characterized by predisposition to gastrointestinal polyposis, mucocutaneous melanin pigmentation, and various neoplasms (1). The incidence of cancer among patients with PJS has been estimated to be 18-fold higher than in the general population (1). Moreover, tumors associated with PJS have acquired somatic mutations in the remaining wild-type allele of *LKB1*, strongly implicating *LKB1* as a tumor susceptibility gene (2). Most of the mutations in PJS families are point and truncation mutations within the kinase domain of *LKB1*, suggesting that the kinase activity of *LKB1* is critical to its function (3, 4).

LKB1 has a tumor suppressor function in the human cancers, including breast cancer (5, 6). In our previous study, we reported that overexpression of *LKB1* protein in MDA-MB-435 cancer cells can result in growth inhibition, which is mediated by G_1 arrest and $p21^{WAF1/CIP1}$ induction, and low expression of the *LKB1* protein in human breast cancer is significantly associated with a shorter survival (5). To further investigate whether overexpression of *LKB1* affects the invasion and metastasis of cancer cell, we generated an *LKB1* high-expression cell line of MDA-MB-435 cancer cells. We showed that *LKB1* exerted tumor inhibitory effects on human carcinoma cells both *in vivo* and *in vitro*. The mechanisms of these inhibitory effects include either the up-regulation or the down-regulation of different effector molecules.

Results

Stable Transfection of LKB1 cDNA in MDA-MB-435 Cells

To study *LKB1* expression in human breast cancer, we did reverse transcription-PCR (RT-PCR) and Western blot analyses on MDA-MB-435 cells. As shown in Fig. 1, we found that MDA-MB-435 cells were truly *LKB1*-negative cells. MDA-MB-435 cells were stably transfected with expression vectors carrying human *LKB1* cDNA. Several G418-resistant clones were tested for the integration of the *LKB1* expression vector into the transfectants. As shown in Fig. 1, two *LKB1* transfectants (clone 1 and clone 2) expressed both higher *LKB1* mRNA and protein. In this study, both clones were used to make further experiments.

Received 5/1/06; revised 8/29/06; accepted 8/31/06.
Grant support: National Natural Science Foundation of China Outstanding Young Investigator Award 30025015, National Key Project of China grant 2001BA703BO5, National Natural Science Foundation of China grant 30371580, and Shanghai Science and Technology Committee grant 03J14019.
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Note: Z-G. Zhuang and G-H. Di contributed equally to this work.
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doi:10.1158/1541-7786.MCR-06-0118

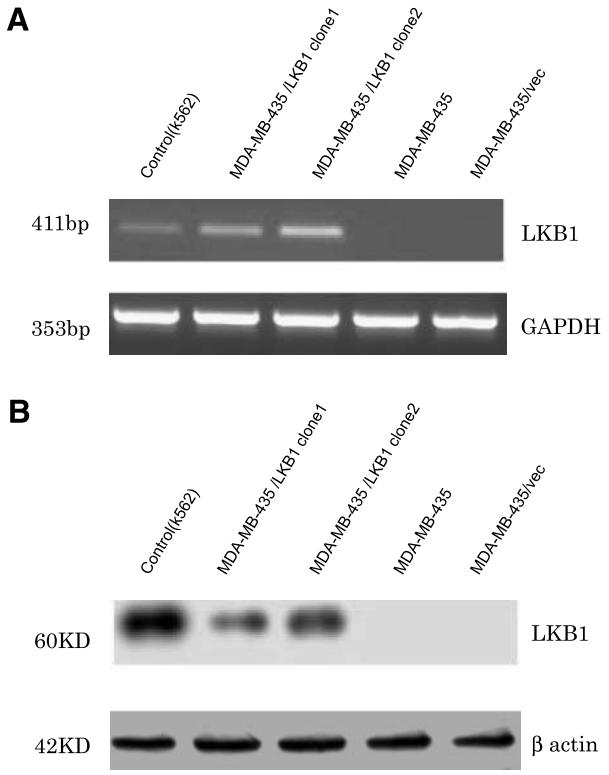

FIGURE 1. *LKB1* expression in the breast cancer cell line MDA-MB-435, vector-transfected MDA-MB-435, and *LKB1*-transfected MDA-MB-435. **A.** *LKB1* mRNA expression in the above three MDA-MB-435 clones (RT-PCR). Lane 1, control (K562); lane 2, MDA-MB-435/LKB1 (clone 1); lane 3, MDA-MB-435/LKB1 (clone 2); lane 4, MDA-MB-435; lane 5, MDA-MB-435/vector (*MDA-MB-435/vec*). **B.** *LKB1* protein expression in the above three MDA-MB-435 clones (Western blot). Lane 1, control (K562); lane 2, MDA-MB-435/LKB1 (clone 1); lane 3, MDA-MB-435/LKB1 (clone 2); lane 4, MDA-MB-435; lane 5, MDA-MB-435/vector.

LKB1 Inhibition of Invasion and Modulation of Relevant Effector Molecules In vitro

It was of interest to investigate whether *LKB1* overexpression might affect the ability of cell motility. To address this issue, we did an invasion study using the classic Transwell *in vitro* assay. As shown in Fig. 2, *LKB1*-transfected MDA-MB-435 cells resulted in a significant decrease of invasion when compared with mock-transfected and wild-type cells, 58% decrease for clone 1, 55% decrease for clone 2. To further study the potential mechanisms of *LKB1* inhibition of invasion, we examined the relevant effector molecules, including matrix metalloproteinases (MMP), basic fibroblast growth factor (bFGF), and vascular endothelial growth factor (VEGF). By RT-PCR and Western blot, *LKB1*-transfected MDA-MB-435 cells constitutively expressed low levels of MMP-2 and MMP-9 mRNA (data not shown) and protein (Fig. 3). By direct gelatin zymography, *LKB1*-transfected MDA-MB-435 cells constitutively secreted low levels of MMPs, 1.8-fold lower compared with mock-transfected or wild-type cells (Fig. 3C and D). For angiogenesis factors, we showed that *LKB1*-transfected MDA-MB-435 cells constitutively expressed significantly lower levels of VEGF and bFGF both in mRNA and protein levels when compared with mock-transfected and wild-type cells ($P < 0.05$; Fig. 3A and B).

LKB1 Inhibition of Tumor Growth, Tumor Metastasis, and Modulation of Relevant Effector Molecules In vivo

LKB1-transfected MDA-MB-435 cells were injected into the mammary fat pad of nude mice to assess the effect of LKB1 *in vivo*. *LKB1*-transfected MDA-MB-435 cells (*LKB1* group) grew much slower than mock-transfected or wild-type cells in nude mice ($P < 0.05$; Fig. 4A). When studying the pulmonary metastasis, it was clearly shown that there was ~70% decrease of lung metastasis in both *LKB1* groups compared with that in the mock-transfected or wild-type group ($P < 0.05$; Fig. 4B and C). Similar effects of *LKB1* on MMPs and VEGF and bFGF expression noted in the *in vitro* studies were also observed in the *in vivo* studies (Fig. 5). By Western blot, we showed that *LKB1*-transfected MDA-MB-435 cells expressed 55% lower levels of MMP-2, 45% lower levels of MMP-9, 67% lower levels of VEGF, and 52% lower levels of bFGF when compared with mock-transfected and wild-type cells ($P < 0.05$).

Higher LKB1 Expression Association with the Decreasing of Microvessel Density and Down-Regulation of VEGF and bFGF Expression in In vivo *Models and in Human Breast Cancer Cases*

We examined the microvessel density (MVD), VEGF, and bFGF expression in paraffin-embedded tissue sections from *LKB1*-transfected MDA-MB-435, mock-transfected, or wild-type cells in nude mice. We found that there is an ~50% decrease of MVD in clone 1 and clone 2 and down-regulation of VEGF and bFGF expression in *LKB1*-transfected MDA-MB-435 cells when compared with mock-transfected or wild-type cells in nude mice ($P < 0.05$; Fig. 6A and B). We further examined paraffin-embedded tissue sections from 38 cases of human breast cancer samples to identify the correlation that we found *in vivo*. As shown in Table 1, there is a strong negative correlation between MVD and *LKB1* expression ($P < 0.01$). In addition, we showed that there are strong negative relationships among LKB1 expression, estrogen receptor status, lymph node status, tumor size, and age (Table 1).

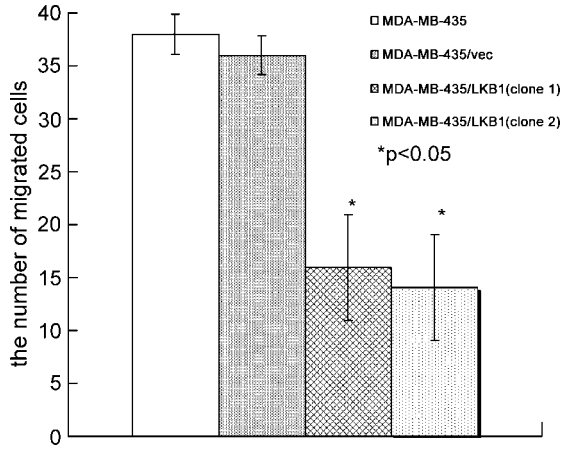

FIGURE 2. Transwell *in vitro* assay. Columns, mean of three independent experiments; bars, SE.

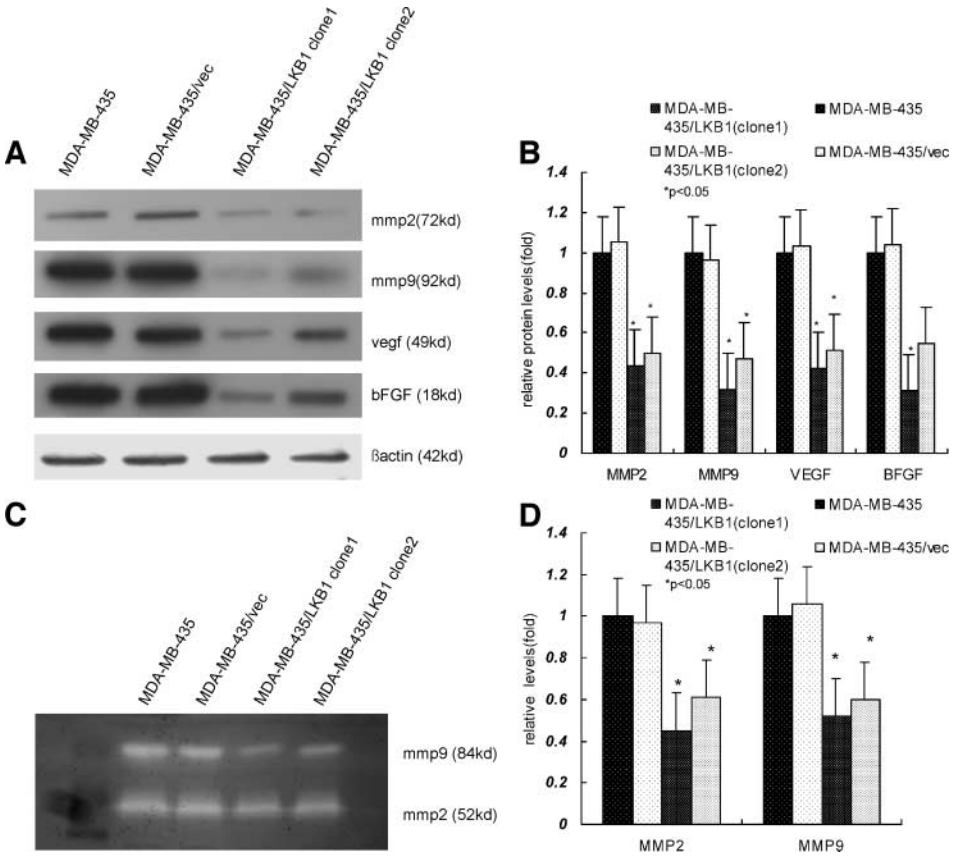

FIGURE 3. **A.** The protein relative levels of MMP-2, MMP-9, VEGF, and bFGF, which were detected by Western blotting in MDA-MB-435, MDA-MB-435/vector, MDA-MB-435/LKB1 (clone 1), and MDA-MB-435/LKB1 (clone 2) cells. **B.** Representative quantitation of three independent experiments. The values of MDA-MB-435 are expressed relative to respective controls (GAPDH), which are given an arbitrary value of 1. Bars, SE. **C.** The relative levels of MMPs were detected by gelatin zymography in MDA-MB-435, MDA-MB-435/vector, MDA-MB-435/LKB1 (clone 1), and MDA-MB-435/LKB1 (clone 2) cells. **D.** Representative quantitation of three independent experiments. The values of MDA-MB-435 are expressed relative to respective controls (GAPDH), which are given an arbitrary value of 1. Bars, SE.

Discussion

LKB1 has been implicated as a tumor suppressor gene, but only few mutations in the coding exons of *LKB1* have been detected in sporadic tumors, including human breast cancer (7). In our previous study, we observed a suggested tumor-suppressive function for *LKB1* in human breast cancer in human cancer cell lines and human breast cancer specimens. We showed that overexpression of the *LKB1* protein results in growth inhibition of tumor cells, which is mediated through G_1 cell cycle arrest, and low expression of the *LKB1* protein in human breast cancer is significantly associated with a shorter survival (5). In the present study, we further showed that *LKB1* inhibits the migration and invasion of MDA-MB-435 cells both *in vitro* and *in vivo* study. In *in vivo* study, we also showed that *LKB1*-transfected MDA-MB-435 cells grew much slower than mock-transfected or wild-type cells in nude mice. On comparing the pulmonary metastasis, it was clearly shown that there was ~2-fold decrease of lung metastasis in *LKB1*-transfected MDA-MB-435 xenografts when comparing mock-transfected or wild-type xenografts. All these were related to down-regulation of MMP-2 and MMP-9 levels. MMPs play important roles in tumor growth, invasion, and metastasis. MMPs are an important group of zinc enzymes that are responsible for degradation of extracellular matrix components, such as collagen and proteoglycans, in normal embryogenesis and in many disease processes, such as arthritis and invasion by carcinoma (8, 9). In our study, we showed that MMP-2 and MMP-9 mRNA and protein expressions were significantly down-regulated in *LKB1*-transfected MDA-MB-435 cells both *in vitro* and *in vivo*. This may also contribute to the inhibition of tumor growth and metastasis.

Angiogenesis is essential for the growth and metastasis of solid tumors (10). Among the many reported angiogenic factors, VEGF and bFGF are the most powerful endothelial cell–specific mitogens associated with tumor neovascularization (11, 12). In the present study, we showed that *LKB1*-transfected MDA-MB-435 cells constitutively expressed significantly lower levels of VEGF and bFGF both in mRNA and protein levels when compared with mock-transfected or wild-type cells both *in vitro* and *in vivo* experiments. Recently, Ylikorkala et al. (13) reported that the mice with a targeted disruption of *LKB1* die at midgestation, with the embryos showing neural tube defects, mesenchymal cell death, and vascular abnormalities. These phenotypes

were associated with tissue-specific deregulation of VEGF expression, including a marked increase in the amount of VEGF mRNA. These findings place *LKB1* in the VEGF signaling pathway and suggest that the vascular defects accompanying *LKB1* loss are mediated at least in part by VEGF.

There is a well-established correlation among increased breast tumor MVD, angiogenesis, and reduced prognosis (14-16). In our system, we first reported that overexpression of *LKB1* resulted in the decrease of MVD both in *in vivo* experiments and in human breast cancer samples. This decrease of MVD was associated with down-regulation of VEGF and bFGF expression.

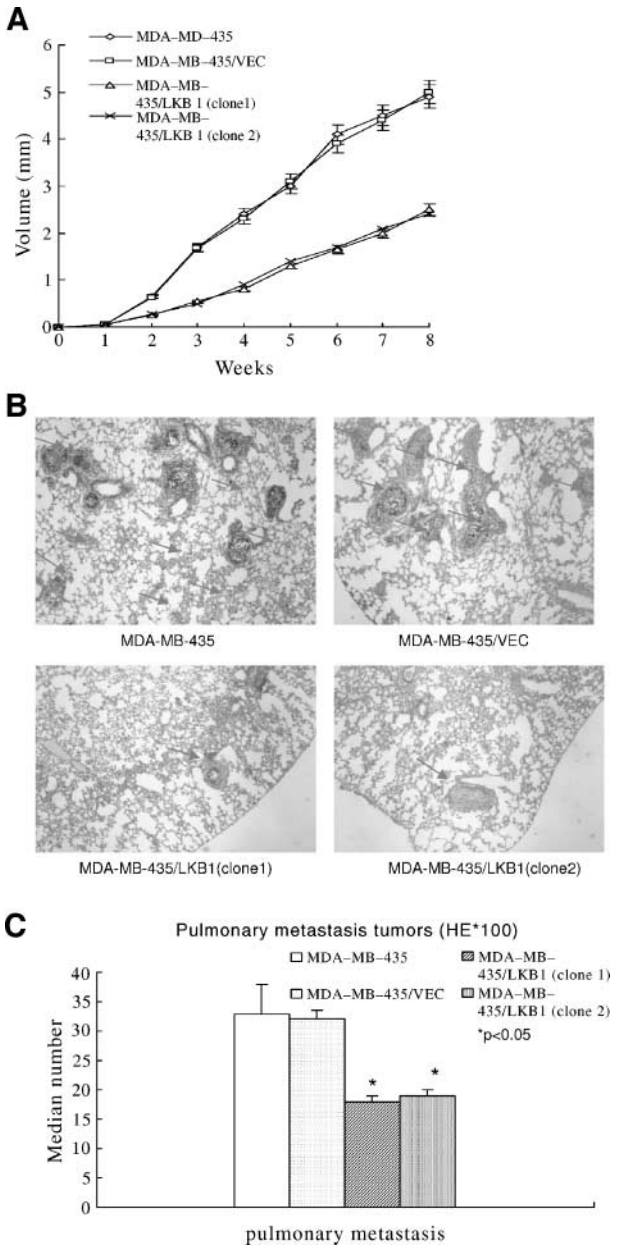

FIGURE 4. **A.** The growth curve of these xenografts in *in vivo* proliferation assay. Points, mean of three independent experiments; bars, SE. **B.** Pulmonary metastasis (*arrows*) was shown in MDA-MB-435, MDA-MB-435/vector, MDA-MB-435/LKB1 (clone 1), and MDA-MB-435/LKB1 (clone 2) cells. H&E, ×100. **C.** Representative tumor metastasis numbers per lung in these four cell lines at the 8th week. Columns, mean of three independent experiments; bars, SE.

Recently, Chung et al. (17) reported that inactivation of the *PTEN* gene protein product is associated with the invasiveness and metastasis of breast cancer. *PTEN* is a novel tumor suppressor gene located on chromosomal band 10q23. Loss of PTEN function has been implicated in the progression of several types of cancer. *PTEN* is known as an *LKB1*-interacting protein (18). Several *LKB1* point mutations associated with PJS disrupt the interaction with PTEN, suggesting that the loss of this interaction might contribute to PJS. Boudeau et al. (19) reported that overexpression of *LKB1* in A549 cells resulted in a significant increased *PTEN* mRNA expression. In our study, we showed that overexpression of *LKB1* in MDA-MB-435 cells resulted in slow-growing tumors and decreasing of lung metastasis in nude mice.

In conclusion, our results indicated that *LKB1* plays a negative regulatory role in human breast cancer. Overexpression of *LKB1* protein in cancer cells can result in significant inhibition of invasiveness and lung metastasis *in vivo*. These inhibitions were associated with down-regulation of MMP-2, MMP-9, VEGF, and bFGF mRNA and protein levels and decreased MVD in xenograft tumors and human breast cancer samples. Overexpression of the *LKB1* protein in human breast cancer is significantly associated with tumor MVD. This finding may lead to a new therapeutic strategy against breast cancer.

Materials and Methods

Construction of the LKB1 Expression Plasmid

The expression plasmid *pcDNA3.1/LKB1* myc, which contains the wild-type *LKB1* coding sequence, was constructed by PCR. A DNA fragment amplified by PCR, by use of an LKE1 primer (5′-GATGAATTCGGGTCCAGCATGGAGG-TGGTGGAC-3′) and an LKE2 primer (5′-GATGAATTCTTA-GAGGTCTTCTTCTGAGATGAGCTTCTGCTCCTGCTG-CTTGCAGGCCGA-3′), was cloned into an *Eco*RI site of the pcDNA3 vector. The clones with the correct orientation were selected, and their sequences were verified.

Cell Culture and Transfections

Estrogen receptor–negative breast cancer cell line MDA-MB-435 was grown in MEM supplemented with 10% (v/v) fetal bovine serum and antibiotics (100 units/mL penicillin and 100 μg/mL streptomycin). These cells were cultured as monolayers in a 95% air, 5% CO_2 water-saturated atmosphere. RT-PCR and Western blot have shown MDA-MB-435 cells lacking the *LKB1* gene (Fig. 1). Then, MDA-MB-435 cells were transfected with *pcDNA3.1/LKB1* expression vector with the LipofectAMINE method (Life Technologies, Rockville, MD). The transfected cells were subjected to 1 mg/mL G418 (Life Technologies) selection. Several G418-resistant clones were tested for the integration of the *LKB1* expression vector into the transfectants. As shown in Fig. 1, two LKB1 transfectants (clone 1 and clone 2) that expressed both higher LKB1 mRNA and protein, which we used in our previous study (5), were further used in this project.

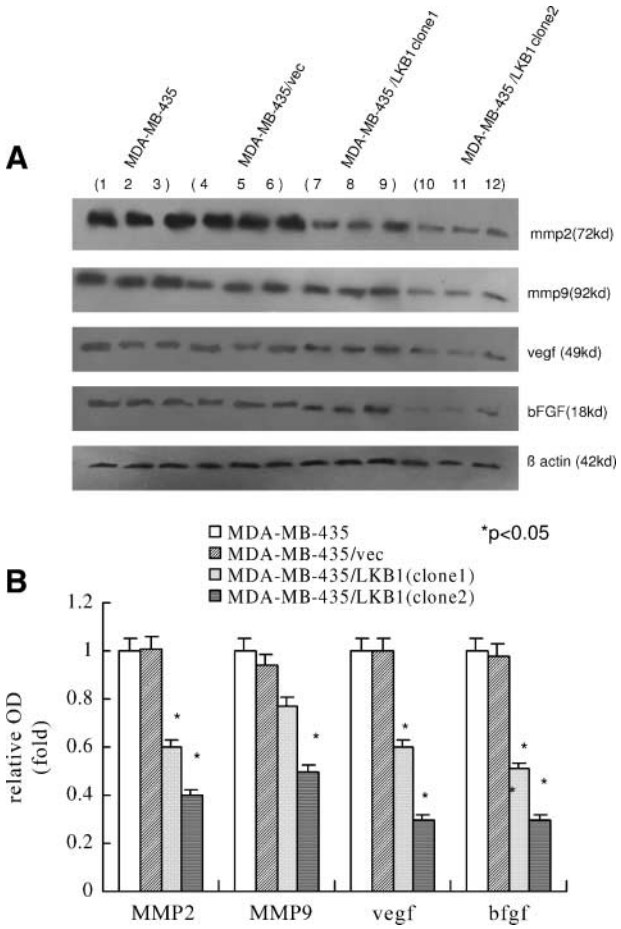

FIGURE 5. **A.** The protein levels of MMP-2, MMP-9, VEGF, and bFGF that were detected by Western blotting in MDA-MB-435 (*lanes 1-3*), MDA-MB-435/vector (*lanes 4-6*), MDA-MB-435/LKB1 (clone 1; *lanes 7-9*), and MDA-MB-435/LKB1 (clone 2; *lanes 10-12*) xenografts. **B.** Representative quantitation of three independent experiments. The values of MDA-MB-435 are expressed relative to respective controls (GAPDH), which are given an arbitrary value of 1. Bars, SE.

Isolation of RNA and RT-PCR

Total RNA was extracted with Trizol (Life Technologies). RNA (0.8 μg) was used in the reverse transcription reaction. The standard random priming method with Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI) and RNase inhibitor (Promega) was used to obtain 20 μL cDNA. PCR was carried out in a volume of 50 μL containing 3 μL cDNA, 1× PCR buffer (Promega), 2.5 mmol/L $MgCl_2$, 200 μmol/L of each deoxynucleotide triphosphate (Promega), each primer at 0.8 μmol/L, 10% DMSO, and 2 units polymerase (Promega). Cycling conditions were done as described previously (20). The PCR products were electrophoresed on 1.5% agarose gel and imaged on a ChemiImage 5500 Imaging System (Alpha Innotech, San Leandro, CA). Densitometry of images was done using NIH Image version 1.62. The values of MDA-MB-435 are expressed relative to respective controls [glyceraldehyde-3-phosphate dehydrogenase (GAPDH)], which are given an arbitrary value of 1 (Table 1). The specific primers and their annealing temperatures are listed in Table 2.

Western Blotting

Cells were washed twice with ice-cold PBS, scraped into 1 mL of ice-cold NP40 lysis buffer [10 mmol/L Tris-HCl (pH 8.0), 150 mmol/L NaCl, 1% (v/v) NP40, 1 mmol/L EDTA, 50 mmol/L NaF, 5 mmol/L sodium pyrophosphate, 1 mmol/L phenylmethyl sulfonyl fluoride, 1 μg/mL leupeptin, 1 μg/mL pepstatin A]. Cells were then sonicated for 5 seconds at 5 W. Insoluble debris was removed by centrifugation at 1,000 × *g* for 15 minutes. Total

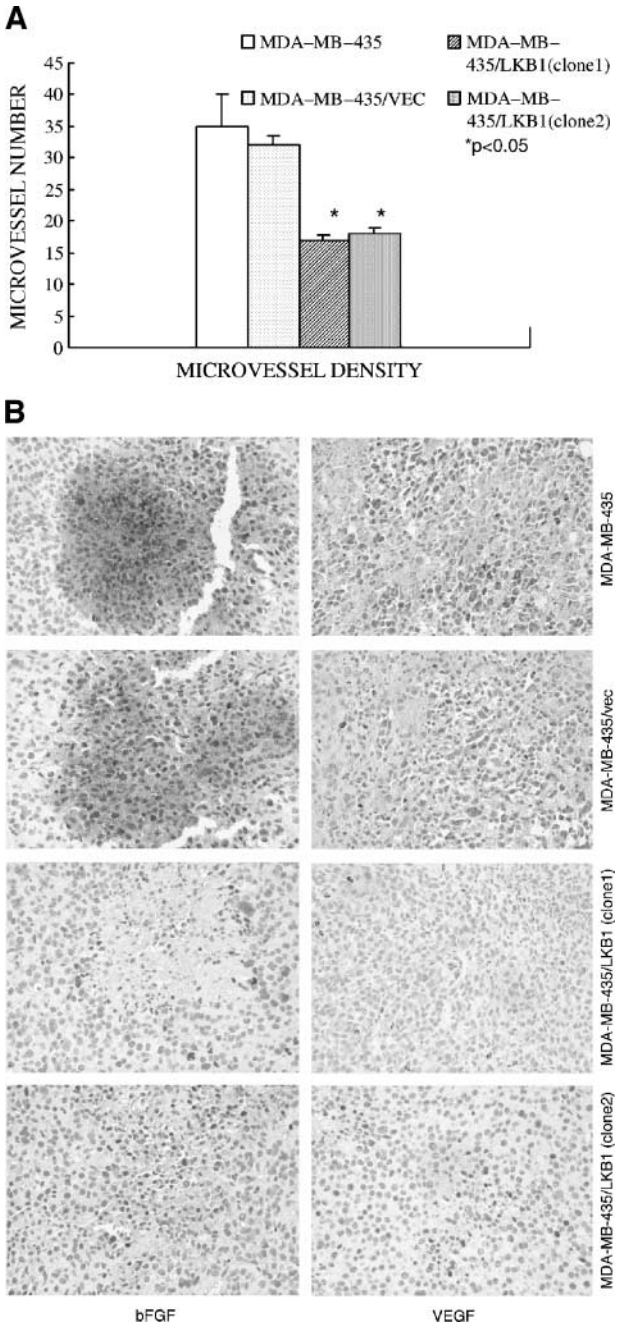

FIGURE 6. **A.** The correlation between LKB1 expression and MVD. **B.** The relationship among LKB1 expression and VEGF and bFGF expression. Representative pictures of immunostaining for bFGF and VEGF. The MVD was detected by labeling CD34 expressed in human breast cancer.

Table 1. The Relationship among LKB1 Status, MVD, and Other Factors

	LKB1		*P*
	Negative	Positive	
MVD status			
+- - - -++	3	12	
+++- - - - -++++	19	4	0.000
Age (y)			
<50	16	5	
≥50	4	13	0.003
Tumor size (cm)			
<3	4	11	
≥3	18	5	0.003
Node status			
Negative	3	14	
Positive	19	2	0.000
Estrogen receptor status			
Negative	15	2	
Positive	7	14	0.001
Age (y)			
<50	16	5	
≥50	4	13	0.003

proteins (200 μg) were analyzed by 10% SDS-PAGE. Western blot using murine monoclonal antibodies to MMPs, VEGF, and bFGF (Oncogene Research Products, Cambridge, MA) and *LKB1* polyclonal antibody (Upstate Biotechnology, Lake Placid, NY) was done according to standard protocols (21). Blot quantitation was done with a Molecular Dynamics laser densitometer (Model PSD, Mount Holly, NJ) and the ImageQuant version 1 software.

In vitro *Assays of Invasion*

Invasion experiments were conducted with a Matrigel invasion chamber (BD Labware, Bedford, MA; Albini, 1998). Each well insert was layered with 120 mL of 1:3 mixture Matrigel-MEM (1,400 mg Matrigel/cm^2). An amount of 10^5 cells was added to the top of this Matrigel layer. The wells were incubated at 37°C for 36 hours. Invasion was assessed by counting the cells that had traveled across the filter and were attached to the bottom side of the filter. Then, the filters were fixed in 10% formalin and stained with H&E. Cells that had invaded through the Matrigel and reached the lower surface of the filter were counted under a light microscope at a magnification of ×200. Five fields should be counted for each sample.

Gelatin Zymography

For gelatin zymography, cell cultures were maintained to 80% confluency in 10% cosmic calf serum DMEM/F12. Then, cells were incubated under serum-free conditions for 24 hours. At the time of medium collection, the cells were counted for the purpose of adjusting the volume of the medium to the cell number. MMP-2 and MMP-9 activity was assessed using 10% zymogram gels (Bio-Rad, Richmond, CA). After electrophoresis, gels were rinsed in 2.5% Triton X-100 for 30 minutes and then enzyme degradation was done at 37°C for 40 hours in 100 mmol/l Tris (pH 7.5), 5 mmol/l $CaCl_2$, and 0.04% NaN_3. The gels were stained with 2.5% Coomassie blue and air dried. Densitometry was used to analyze relative MMP-9 activity with NIH Image version 1.62.

In vivo *Study with Animal Experiment*

Female athymic BALB/c *nu/nu* mice, 4- to 6-week old, were obtained from the Shanghai Institute of Material Medica, Chinese Academy of Sciences (Shanghai, China), and housed in laminar flow cabinets under specific pathogen-free condition. All studies on mice were conducted in accordance with the NIH 'Guide for the Care and Use of Laboratory Animals'. The study protocol was approved by the Shanghai Medical Experimental Animal Care Committee. The tumorigenicity and spontaneous metastatic capability of the cell lines were determined by injection into the mammary fat pad. In total, 1×10^6 cells in 0.1 mL of culture medium were inoculated into the anesthetized mouse. Animals were divided into four groups: MDA-MB-435, MDA-MB-435/vector, MDA-MB-435/LKB1 (clone 1), and MDA-MB-435/LKB1 (clone 2). Each group had six mice. Animals were monitored every 2 days for up to 8 weeks for tumor growth and general health. The rate of primary tumor growth of different cells was determined by plotting the means of two orthogonal diameters of the tumors, measured at 7-day intervals. Animals were killed and autopsied at 8 weeks after inoculation. The lungs that were used to evaluate the numbers of metastasis were fixed in Bouin's liquid for 24 hours at first. Then, they were stored in 100% ethanol. When the lungs restored their inherent color, the metastasis deposits with white color could be assessed by macroscopic observation. To confirm the presence of lung metastases, sections were cut at 50-mm intervals and H&E staining was done. In this study, we count metastasis in whole lung. Two independent pathologists calculated the numbers of metastasis. Tissue samples were harvested for and embedded in paraffin wax or snap frozen in liquid nitrogen (Urquidi et al., 2002).

Immunohistochemical Detection of CD34 and LKB1

Tumor sections were subjected to immunohistochemical staining for CD34 and *LKB1*. Tumor sections were incubated in a 1:200 dilution of mouse anti-human CD34 and *LKB1* (Santa Cruz Biotechnology, Santa Cruz, CA) serum or mouse preimmune serum (used on human breast cancer sections) and 1:10 dilution of rat anti-mouse CD34 serum or rat preimmune serum. Primary antibody was detected with biotinylated antibodies followed by incubation with streptavidin-conjugated horseradish peroxidase and colorimetric detection with 3,3′-diaminobenzidine. All immunostained tissue sections were evaluated in a coded manner without knowledge of the clinical or pathologic variables. CD34 and *LKB1*-stained sections were

Table 2. Specific Primers and Their Annealing Temperatures

LKB1	Up	5′-CGGCAAGGTGAAGGAA-3′		
	Down	5′-ACGCCCAGGTCGGAGAT-3′	411 bp	55°C
MMP-2	Up	5′-CAGGCTCTTCTCCTTTCACAAC-3′		
	Down	5′-AAGCCACGGCTTGGTTTTCCTC-3′	398 bp	55°C
MMP-9	Up	5′-TGGGCTACGTGACCTATGACAT-3′		
	Down	5′-GCCCAGCCCACCTCCACTCCTC-3′	172 bp	60°C
VEGF	Up	5′-CTACCTCCACCATGCCAAGT-3		
	Down	5′-TCTCTCCTATGTGCTGGCCT-3′	311 bp	60°C
bFGF	Up	5′-AGAGCGACCCTCACATCAAG-3′		
	Down	5′-ACTGCCCAGTTCGTTTCAGT-3′	234 bp	60°C
GAPDH	Up	5′-GGGAGCCAAAAGGGTCATCATCTC-3′		
	Down	5′-CCATGCCAGTGAGCTTCCCGTTC-3′	353 bp	60°C

Table 3. Patient Demographics

	No. patients (N = 38)
Age (y)	
Median	50.3
Range	33-68
<50	21
≥50	17
Surgical treatment	
Lumpectomy	7
Lumpectomy + radiotherapy	16
Mastectomy	15
Tumor size (cm)	
<3	15
≥3	23
Node status	
Negative	17
Positive	21
Histologic type	
Ductal	27
Lobular	5
Medullary	2
Adenocarcinoma	3
Others	1
Estrogen receptor status	
Negative	17
Positive	21
Progesterone receptor status	
Negative	19
Positive	19

scanned at low magnification (×40) to determine areas with the highest number of microvessels (hotspots). Microvessels whose diameters were less than that of eight red cells were counted at a magnification of ×200 in two hotspots on each section, and MVD was calculated as the average of the two measurements. MVD was semiquantitatively rated on a four-grade scale (+, ++, +++, and ++++) according to the hotspots and further divided into two groups, low MVD (+ and ++) and high MVD (+++ and ++++), for statistical analysis.

Human Breast Cancer Sample Collection and Protein Preparation

Thirty-eight patients with histologically confirmed local breast carcinoma were included in the present study. This study was done in the Cancer Hospital of the Shanghai Medical University between March 2003 and March 2004. All the patients came from the Cancer Hospital of the Fudan University. The patient's characteristics were shown in Table 3. The protocol of this study was approved by the human research committees of the Cancer Hospital and informed consent was obtained from each patient. All patients were followed up to determine their clinical outcome.

Statistical Analysis

ANOVA and Student's t test were used to determine the statistical significance of differences among experimental groups.

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