**Advances in Brief**

**The Tumor Suppressor Gene LKB1 Is Associated with Prognosis in Human Breast Carcinoma**

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**Abstract**

*Purpose:* LKB1 (also called STK11) is a recently identified tumor suppressor gene in which its mutation can lead to Peutz-Jeghers syndrome, characterized by gastrointestinal polyps and cancers of different organ systems. Weak expression of this gene does occur at a certain frequency in sporadic breast cancer. This indicates that LKB1 gene may relate to the tumorigenesis of breast cancer.

*Experimental Design:* To investigate the function of the LKB1 gene in sporadic breast cancer, we reintroduced LKB1 into breast cancer cell lines which lack the LKB1 gene. Also, we examined the LKB1 protein expression in human breast cancer samples.

*Results:* We found that reintroducing LKB1 into breast cancer cell lines suppresses cell growth by G1 cell cycle block. The LKB1-mediated G1 cell cycle arrest is caused by up-regulation of the expression of p21WAF1/CIP1 in breast cancer MDA-MB-435 cells. We also demonstrated that low LKB1 protein expression correlates with higher histological grade (P = 0.013), larger tumor size (P = 0.001), progesterone receptor status (P = 0.048), and presence of lymph node metastasis (P = 0.003). Furthermore, LKB1 low expression was associated with a higher relapse rate (P = 0.002) and a worse OS (P = 0.008).

*Conclusions:* LKB1 plays a role in tumor suppressor function in human breast cancer. LKB1 expression may be a useful prognostic marker in human breast cancer.

**Introduction**

The LKB1/STK11 gene (focus on 19p13.3) encodes a serine/threonine protein kinase and is deficient in the majority of patients with PJS (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 (1, 3).

Because PJS patients show a predisposition to a wide spectrum of cancers, including breast cancer, it is speculated that LKB1 has a tumor suppressor function in the breast cancer of PJS. A report of 31 sporadic breast cancers showed weak LKB1 expression in 9 cases of cancer (4). We ask that if the LKB1 gene has a role in breast cancer malignant formation, what is the mechanism of this tumor suppressor function? To investigate the function and the mechanism of LKB1 in sporadic breast cancer, we have identified breast cancer cell lines with severely reduced LKB1 mRNA and protein expression and transfected the LKB1 gene into one of these, the MDA-MB-435 cells. We found that the LKB1 overexpression can result in G1 cell cycle arrest, which is accompanied by p21WAF1/CIP1 induction. We also investigated LKB1 protein expression in 116 cases of breast cancer samples and demonstrated that low LKB1 protein expression correlated with higher histological grade, larger tumor size, presence of lymph node metastasis, and shorter survival.

**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'-GATGAATTCGGGTCCAGCATGGAGGTGGTGGAC-3') and an LKE2 primer (5'-GATGAATTCAGAGGTCTCTCTTCGTGAGATGAGCTTCTCTGCTGTCCTCTGTCGAGGCGCA-3'), was cloned into an EcoRI site of the pcDNA3 vector. The clones with the correct orientation were selected, and their sequences were verified.

**Cell Culture and Transfections.** ER-positive breast cancer cell line MCF-7 and ER-negative breast cancer cell lines MDA-MB-435 and MDA-MB-231 were 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 95% air, 5% CO2 saturated atmosphere. RT-PCR and Western blot have shown MDA-MB-435 cells that lack the LKB1 gene (Fig. 1 in “Results”). Then, MDA-MB-435 cells were transfected with pcDNA3.1/LKB1 expression vector with the lipofectAMINE method (Life Technologies, Inc., Rockville, MD). The trans-
fected cells were subjected to 1 mg/ml G418 (Life Technologies, Inc.) selection.

Isolation of RNA and RT-PCR. Total RNA was extracted with Trizol (Life Technologies, Inc.). An amount of 0.8 µg of RNA was used in the reverse transcription reaction. The standard random priming method with Moloney murine leukemia virus reverse transcripts (Promega) and RNase inhibitor (Promega, Madison, WI) was used to obtain 20 µl of cDNA. The following primers were used to amplify most of the coding region of LKB1: LKBF1, 5'-GAGCTGATGTCGGTGCTC-3'; and LKB5, 5'-GCCCTGATTGTTTGCTC-3'. PCR was carried out in a volume of 50 µl containing 3 µl of cDNA, 1X PCR buffer (Promega), 2.5 mM MgCl2, 200 µM of each deoxynucleotide triphosphate (Promega), each primer at 0.8 µM, 10% DMSO, and 2 units of polymerase (Promega). Cycling conditions were performed as described previously (5).

Western Blotting. Cells were washed twice with ice-cold PBS and scraped into 1 ml of ice-cold NP40 lysis buffer [10 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1% (v/v) NP40, 1 mM EDTA, 50 mM NaF, 5 mM NaPPi, 1 mM phenylmethylsulfonyl fluoride, 1 µg/ml leupeptin, and 1 µg/ml pepstatin A]. Cells were then sonicated for 5 s at 5 W. Insoluble debris was removed by centrifugation at 1000 × g for 15 min. Total proteins (200 µg) were analyzed by 10% SDS-PAGE. Western blot, using murine monoclonal antibodies to p21WAF1/CIP1 (Oncogene Research Products, Cambridge, MA), cyclin D1 and cyclin E (Santa Cruz Biotech, San Diego, CA), and LKB1 polyclonal antibody (Upstate Biotechnology, Lake Placid, NY), was performed according to standard protocols (6, 7). Blot quantitation was done with a Molecular Dynamics Laser Densitometer (Model PSD) and the Image Quant Version 1 software.

Analysis of Cell Cycle Phase Distribution. Flow cytometry analysis of DNA content was performed to assess the cell cycle phase distribution as described previously (6). Cells were harvested and stained for DNA content using propidium iodide fluorescence. The computer program Multicycle from Phenix Flow System (San Diego, CA) was used to generate histograms, which were used to determine the cell cycle phase distribution.

Human Breast Cancer Samples Collection and Protein Preparation. A total of 116 patients with histologically confirmed local breast carcinoma was included in this study. This study was performed at the Cancer Hospital of the Shanghai Medical University between March 1995 and December 2000. All of the patients were from the Cancer Hospital of the Shanghai Medical University. The mean age is 53.7 years old, and the clinical data are shown in Table 1. 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.

Tumor specimens were obtained at surgery and immediately frozen in liquid nitrogen for protein extraction and assay of ERs. Routine fixation in formalin and paraffin wax embedding was done for histological assessment. Histological types were determined according to the WHO criteria (8). Protein extraction from tumor and tissue samples was performed as described previously (6).

<table>
<thead>
<tr>
<th>Patient demographics</th>
<th>No. of Patients (N = 116)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yrs.)</td>
<td>Median: 53.7, Range: 32–77</td>
</tr>
<tr>
<td>Node status</td>
<td>Negative: 46, Positive: 75</td>
</tr>
<tr>
<td>Histological type</td>
<td>Ductal: 96, Lobular: 8, Medullary: 7, Paget’s disease: 1, Adenocarcinoma: 5, Others: 4</td>
</tr>
<tr>
<td>Grade</td>
<td>1: 17, 2: 57, 3: 47</td>
</tr>
<tr>
<td>ER status</td>
<td>Positive: 78, Negative: 43</td>
</tr>
<tr>
<td>PR status</td>
<td>Positive: 70, Negative: 51</td>
</tr>
<tr>
<td>Adjuvant therapy</td>
<td>Chemotherapy: 56, Hormonal therapy (tamoxifen): 43</td>
</tr>
<tr>
<td>Duration follow-up (mo)</td>
<td>Median: 44.5, Range: 10–70</td>
</tr>
<tr>
<td>Relapses</td>
<td>26</td>
</tr>
<tr>
<td>Deaths</td>
<td>15</td>
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</tbody>
</table>

Patient Follow-up. All patients were followed up after surgical treatment. Physical examination was performed every 3 months in all patients for the first 2 years and then twice/year. Mammography, radiographic studies (chest X-ray), and liver ultrasounds were performed every 12 months; bone scans and computed tomography scans were performed whenever clinically indicated. Blood tests, including electrolyte and liver function profiles and complete blood cell counts, were repeated at every follow-up visit. RFS was calculated as the period from surgery until the date of the first recurrence.

Statistical Analysis. LKB1 was evaluated in relation to a range of established prognostic variables by the Mann-Whitney test and the Kruskal-Wallis tests. The association with RFS and OS was assessed by univariate analysis (log-rank test and Kaplan-Meier method).

Results

Absence of LKB1 Expression in Human Breast Cancer Cell Lines. To study LKB1 expression in human breast cancer, we performed RT-PCR and Western blot analyses on a
panel of human breast cancer cell lines, including MDA-MB-435, MDA-MB-231, and MCF-7. As shown in Fig. 1, we found that ER-negative breast cancer cells MDA-MB-435 and MDA-MB-231 had undetectable levels of LKB1 expression, whereas ER-positive MCF-7 cells had a relative high LKB1 expression.

Reintroducing LKB1 into MDA-MB-435 Cells Restores LKB1 Activity and Induces Growth Suppression. Down-regulation of LKB1 expression in MDA-MB-435 cells may have provided a growth advantage to these cells. To investigate this possibility, we transfected an LKB1 expression vector (LKB1/pcDNA3-neo) into MDA-MB-435 cells and generated stable transfection of cell lines. LKB1 was detected by RT-PCR and Western blotting. As shown in Fig. 2, the level of LKB1 expression detected in the LKB1-transfected MDA-MB-435 cells was almost as high as that observed in normal liver tissue that was used as positive control. To investigate whether LKB1 expression affects the growth of MDA-MB-435 cells, we performed proliferation experiments comparing LKB1-transfected cells versus vector-transfected cells or wild-type MDA-MB-435 cells. Comparing with those in the vector-transfected cells or wild-type cells, the expressions of cyclin D1 and cyclin E in the LKB1-transfected cells were down-regulated, whereas the p21WAF1/CIP1 expression was up-regulated (Fig. 4).

Expression of LKB1 in MDA-MB-435 Cells Leads to G1 Cell Cycle Arrest. Cell cycle changes in LKB1-transfected cells were examined by flow cytometry. A comparison of G1, S, and G2-M cell cycle phases between vector- and LKB1-transfected cells revealed significant differences (Fig. 3B). The G1 fraction of LKB1-transfected cells was 40.9% compared with 18.3% of vector-transfected cells. This result indicates that LKB1 growth suppression results in G1 cell cycle arrest.

Modulation of the Expression of Cell Cycle-associated Proteins in LKB1 Transfected. To investigate the mechanism of ectopic LKB1 inducing G1 cell cycle arrest in the MDA-MB-435 cells, we examined the expression of cell cycle-associated proteins in the LKB1-transfected and vector-transfected cells or wild-type MDA-MB-435 cells. Comparing with those in the vector-transfected cells or wild-type cells, the expressions of cyclin D1 and cyclin E in the LKB1-transfected cells were down-regulated, whereas the p21WAF1/CIP1 expression was up-regulated (Fig. 4).

The LKB1 Kinase Expression in the Tissues of Breast Cancer. A group of 116 cases of invasive breast cancer patients treated surgically was investigated with Western blot analysis (Fig. 5). Blot quantitation was done with a Molecular Dynamics Laser Densitometer (Model PSD) with Image Quant Version 1 software. Lysate of liver tissue and its blot was set as 1. By using Rank-Log test, the bands of the breast cancer tissue in which its quantities were >0.5 were named as “low expression,” whereas those >0.5 were named as “high expression.”
There were 38 cases showing low \textit{LKB1} protein expression and 73 cases high protein expression.

\textbf{Statistical Analysis}. Between 1995 and 2000, 116 patients with invasive breast cancer were referred for surgical treatment. Median follow-up was 44.5 months, and no patient was lost from follow-up. The information of the patient demographics is listed in Table 1.

The \textit{LKB1} protein expressions in the breast cancer tissue of patients are illustrated in Fig. 5. Low expression of \textit{LKB1} was seen in 38 (31.4\%) cases and 73 (68.4\%) cases showed high expression of \textit{LKB1}. The expression levels of \textit{LKB1} were correlated with several established clinicopathological prognostic variables (Table 2). Within the entire cohort, significant associations were found between the \textit{LKB1} low expression and higher histological grade ($P = 0.013$), tumor size ($P = 0.001$), progesterone receptor status ($P = 0.048$), and presence of lymph node metastasis ($P = 0.003$). There were no apparent relationships with other prognostic variables such as age, tumor histopathological types, and ER status.

Univariate analysis of low expression of \textit{LKB1} showed a significant association with a shorter RFS ($P = 0.008$) and a poorer OS ($P = 0.02$; Fig. 6).

\textbf{Discussion}\n
\textit{LKB1} has been implicated as a tumor suppressor gene, but only few mutations in the coding exons of \textit{LKB1} have been detected in sporadic tumors, including human breast cancer (9). In this study, we have made observations in human breast cancer...
cell lines and cancer specimens that suggest a tumor-suppressive function for \textit{LKB1} in human breast cancer. To demonstrate this possibility, we reintroduced the \textit{LKB1} gene into human breast cancer MDA-MB-435 cells that express undetectable \textit{LKB1} mRNA and protein. We found that overexpression of the \textit{LKB1} protein results in growth inhibition of tumor cells, which is mediated through G1 cell cycle arrest. Tiainen et al. (10) also reported that growth suppression effect by the \textit{LKB1} gene involves G1 cell cycle arrest in the cervical cancer cell lines HeLa S3 and G361. This study and our data suggest that the \textit{LKB1} gene may be involved in a G0-G1 checkpoint for growth inhibition.

The mechanism of growth inhibition of \textit{LKB1} gene remains to be elucidated. However, the G0-G1 checkpoint is tightly related to cell cycle progression, which is regulated by CdKs in association with different cyclins (11–13). Different CdKs-cyclin complexes are required at various stages of cell cycle (cyclin D-CdK4-CdK6 acting in the G1 phase, and cyclin E-CdK2 and cyclin A-CdK2 acting in the G1 and S phase; Refs. 11, 12). Cyclin-CdK activity is regulated by phosphorylation events and cyclin kinase inhibitors, which bind the cyclin-CdK complexes and inhibit their activity (12). The p21WAF1/CIP1 family is an important group of Ckis, including the p21, p27, and p57 proteins, which share partial structural homology and possess the ability to inhibit several cyclin-CdK complexes in vitro, but seem to target preferentially those containing CdK2 (13–15). The p53 tumor suppressor gene is the main factor that regulates p21WAF1/CIP1 (16). Other transcription factors also control p21WAF1/CIP1 expression via p53-independent pathway (17, 18). p53 is a key tumor suppressor gene, which can induce growth arrest, terminal differentiation, or apoptosis (19, 20). The mutations of p53 are much higher than the other tumor suppressor genes and implies that p53 mutant proteins confer some selective advantage in carcinogenesis (15, 21). In this study, we demonstrated that overexpression of the \textit{LKB1} protein in MDA-MB-435 cells can result in an up-regulation of p21WAF1/CIP1 and down-regulation of Cyclin D1 and cyclin E protein expression; these are the possible mechanisms for \textit{LKB1} growth inhibition.

Growth inhibition by \textit{LKB1} may also be associated with the \textit{Brahma-related gene 1} (Brg1) gene. Marignani et al. (7) have demonstrated that \textit{LKB1} binds to and regulates Brg1, an essential component of a chromatin remodeling complex. The association requires the NH2 terminus of \textit{LKB1} and the helicase domain of Brg1 and \textit{LKB1} stimulate the ATPase activity of Brg1. Brg1 expression in SW13 cells induces the formation of flat cells indicative of cell cycle arrest and senescence. Expression of a kinase-dead mutant of \textit{LKB1} in SW13 cells blocks the formation of Brg1-induced flat cells, indicating that \textit{LKB1} is required for Brg1-dependent growth arrest (7). \textit{LKB1} to suppress cell growth is also required at Ser431 by p90RBL and

\begin{table}[h]
\centering
\caption{Association between \textit{LKB1} expression and other prognostic variables}
\begin{tabular}{llll}
\hline
Prognostic parameter & \textit{LKB1} Low & \textit{LKB1} High & \textit{P} \\
\hline
Progesterone receptor & & & \\
Negative & 21 & 30 & \\
Positive & 17 & 53 & 0.048 \\
Grade & & & \\
1 & 3 & 14 & \\
2 & 13 & 44 & 0.013 \\
3 & 22 & 25 & \\
Histological type & & & \\
Ductal & 32 & 66 & \\
Lobular & 2 & 6 & \\
Medullary & 2 & 5 & 0.186 \\
Paget's disease & 0 & 1 & \\
Adenocarcinoma & 1 & 4 & \\
Others & 1 & 3 & \\
Node & & & \\
Negative & 7 & 39 & 0.003 \\
Positive & 31 & 44 & \\
Size & & & \\
<3 cm & 25 & 73 & \\
\geq 3 cm & 13 & 11 & 0.001 \\
ERα & & & \\
Negative & 14 & 29 & \\
Positive & 24 & 54 & 0.839 \\
\hline
\end{tabular}
\end{table}

Fig. 6 Kaplan-Meier estimates of disease-free survival (A) and OS (B) according to high and low \textit{LKB1} expression in human breast cancer patients.
cAMP-dependent proteinase but not its farnesylation at Cys\(^{433}\) (5). Sapkota et al. (5) reported that the reintroduction of wild-type \(LKB1\) into a cell line that lacks \(LKB1\)-suppressed growth, but mutants of \(LKB1\) in which Ser\(^{431}\) was mutated to Ala to prevent phosphorylation of \(LKB1\) were ineffective in inhibiting growth.

What is the role of \(LKB1\) in human breast cancer patients? In this study, we also examined \(LKB1\) protein expression in 116 cases of human breast cancer samples. We found that in a total of 116 cases, there are 73 (68.4\%) cases showing high expression of \(LKB1\). We also demonstrated that \(LKB1\) protein expression levels are significantly associated with histological grade \((P = 0.013)\), tumor size \((P = 0.001)\), progesterone receptor status \((P = 0.048)\), and presence of the lymph node metastasis \((P = 0.003)\). Furthermore, we demonstrated that low expression of \(LKB1\) protein shows a significant association with a shorter RFS \((P = 0.008)\) and a poorer OS \((P = 0.02\); Fig. 6). To our knowledge, this is the first study that describes a possible prognostic value for low \(LKB1\) expression in human breast cancer. Of course, additional work in larger cohorts of patients is needed to confirm these findings.

In conclusion, our results indicate that \(LKB1\) plays a role in tumor suppressor function in human breast cancer. Overexpression of \(LKB1\) protein in breast cancer cells can result in growth inhibition, which is mediated by \(G_1\) arrest and p21\(^{WAF1/CIP1}\) induction. Low expression of the \(LKB1\) protein in human breast cancer is significantly associated with a shorter survival. \(LKB1\) expression may be a useful prognostic marker in human breast cancer.

References
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