Phe354Leu Polymorphism of \textit{LKB1} Is a Potential Prognostic Factor for Cytogenetically Normal Acute Myeloid Leukemia

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Abstract. Background/Aim: Liver kinase B1 (LKB1) is a major activator of the AMP-dependent kinase/mammalian target of rapamycin pathway. The prevalence and the specificity of LKB1 mutation in acute myeloid leukemia (AML) have not been well established. This study aimed to examine mutation of LKB1 in AML and its clinical and pathological implications. Patients and Methods: Eighty-five patients newly diagnosed with cytogenetically normal AML were analyzed using polymerase chain reaction followed by direct sequencing. Results: A silent mutation (837C>T) of LKB1 was detected in one patient and a pathogenic polymorphism Phe354Leu which diminishes LKB1 ability to maintain cell polarity was detected in six (7%) patients. The Phe354Leu polymorphism occurred concurrently with mutations of nucleophosmin 1 (NPM1), fms-related tyrosine kinase 3 (FLT3) and CCAAT/enhancer binding protein alpha (CEBPA), but not with metabolism-related genes, isocitrate dehydrogenase [nicotinamide adenine dinucleotide phosphate (+)]1 (IDH1) and IDH2. Patients with Phe354Leu polymorphism diagnosed at younger ages had a worse overall survival. Conclusion: LKB1 may be involved in the leukemogenesis and progression of cytogenetically normal AML.

Acute myeloid leukemia (AML) is a very heterogeneous group of leukemia types with diverse presentation and variable responsiveness to therapy (1). Karyotype abnormality represents an important prognostic parameter in AML (2, 3). Nevertheless, approximately 50% of all patients with AML have a normal karyotype and are currently categorized in the intermediate-risk group (1-3). This group is quite heterogeneous, and additional molecular markers for the discrimination between prognostically different subsets of patients is of increasing importance (4). In recent years, several novel molecular markers have been identified that are important for prognostic relevance of patients with AML with normal karyotype (5, 6).

The tumor-suppressor gene liver kinase B1 (LKB1), also known as \textit{STK11}, is located on chromosome 19p13.3 (7). It consists of 11 coding exons and encodes a protein of 436 amino acids with a serine/threonine kinase, and possesses two nuclear localization signals in the N-terminal region, a central catalytic kinase domain and a C-terminal putative farnesylation motif (8). The \textit{LKB1} gene is ubiquitously expressed at varying levels in all fetal and adult tissues, with

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notably higher expression in the pancreas, liver, testes and skeletal muscle (9).

In complex with two other proteins, the STE20-related adapter (STRAD) pseudokinase and the scaffolding protein mouse protein 25 (MO25) (10), LKB1 has been shown to regulate cell-cycle arrest, apoptosis, autophagy and cellular energy metabolism, as well as cell polarity (11-14). LKB1 activates adenosine monophosphate (AMP)-activated protein kinase (AMPK) and other members of the AMPK family (15). The LKB1/AMPK pathway serves as the cellular energy sensor, allosterically activated under low cellular energy conditions by the accumulation of AMP molecules. Activation of AMPK stimulates catabolic pathway such as glycolysis and blocks anabolic pathways such as gluconeogenesis and lipogenesis, and controls protein synthesis though inhibition of the mammalian target of rapamycin (mTOR). The LKB1/AMPK pathway blocks cell growth under low nutrient conditions, and therefore is considered a tumor-suppressor pathway (16).

Germline mutations of the LKB1 gene are responsible for Peutz-Jeghers syndrome, which is an autosomal dominant disorder characterized by hyperpigmentation and multiple benign gastrointestinal hamartomatous polyps. Patients with Peutz-Jeghers syndrome have an increased risk of gastrointestinal and several other types of cancer, including of the pancreas, lung, breast, uterus, cervix, testis and ovary (17). Somatic mutations of the LKB1 gene have also been found in multiple sporadic cancer of the lung, pancreas, ovary, cervical and testis (18, 19). Mice with a heterozygous deletion of Lkb1 are tumor prone, showing an increased incidence of the development of cancer as well as increased susceptibility to carcinogen-induced tumorigenesis (20). Deletion or mutation of the LKB1 gene is associated with a reduced progression-free survival in patients with cervical cancer (21). These observations further indicate a critical role of LKB1 in tumorigenesis and progression.

Several recent studies show that loss of Lkb1 in adult mice leads to loss of hematopoietic stem cell (HSC) quiescence, resulting in depletion of the HSC pool and a marked reduction of HSC repopulating potential in vivo. LKB1-deficient HSCs and bone marrow cell exhibit reduced mitochondrial membrane potential and depletion of cellular ATP. These data define an essential role of the LKB1 in restricting HSC entry into the cell cycle and in maintaining energy homeostasis through AMPK-dependent and AMPK-independent mechanisms (22-24). Moreover, several studies showed that the anti-diabetic drug metformin (an LKB1/AMPK activator) exerted significant anti-leukemia cell activity in AML and T-cell acute lymphoblastic leukemia cells through inhibiting mTOR activity (25, 26). These studies demonstrated that the LKB1/AMPK tumor-suppressor axis is generally functional in hematopoietic cancer and that pharmacological intervention activating this pathway may represent a new target in anticancer therapy (25, 26).

In contrast to the expanding research field on LKB1 in solid tumors, the biological and clinical implications of LKB1 gene alterations in hematological cancers have not been well established. Therefore, we investigated the prevalence and the clinical prognostic significance of LKB1 mutations in patients with newly-diagnosed AML to explore the potential of the LKB1/AMPK signaling pathway as a new target for anticancer drug development of hematologic malignancy.

Materials and Methods

Patient samples. Diagnostic bone marrow samples from 85 de novo adult patients with cytogenetically normal (CN) AML were collected at Kaohsiung Medical University Hospital. Complete remission was defined as the presence of fewer than 5% blasts cells in the bone marrow aspirate examination and evidence of normal maturation of other marrow elements after the first or second course of induction therapy. Only patients with fully regenerated peripheral blood counts (neutrophil recovery to 1.0×10⁹/l and platelets to100×10⁹/l) after induction therapy were included. This study was approved by the Institute Review Board of the Kaohsiung Medical University Hospital (IRB no. KMUH-IRB-990483), and bone marrow samples were obtained with informed consent. Screening of additional molecular markers associated with cytogenetically normal AML, namely fms-related tyrosine kinase 3 (FLT3) internal tandem duplication (FLT3-ITD), FLT3 tyrosine kinase domain (FLT3-TKD) mutation, nucleophosmin 1 (NPM1) mutation, CCAAT/enhancer binding protein alpha (CEBPA) mutation, isocitrate dehydrogenase 1 (IDH1) and IDH2 were conducted as described previously (27-31).

Analysis of LKB1 mutations. To detect the presence of LKB1 mutation, reverse transcription–polymerase chain reaction (RT-PCR) was performed as published previously (32). cDNA was synthesized from 2 μg of total RNA using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). The cDNA sequence of the LKB1 gene was obtained from GenBank [GenBank: NM_000455]. The nine exons of LKB1 gene were conducted as shown in Table I. PCR was carried out in a 25-μl final volume containing approximately 1 μl cDNA, 200 nM of each primers, 200 μM dNTPs, 1.5 mM MgCl₂, 1.25 U GoTaq® Flexi DNA Polymerase (Promega, Madison, WI, USA), and supplied buffer. PCR amplification consisted of initial denaturation at 95˚C for 2 min followed by 35 cycles of 95˚C for 40 sec, 62˚C for 40 sec, and 72˚C for 1 min prior to a final elongation process at 72˚C for 5 min. The PCR products were purified with a QiAquick PCR-purification kit (Qiagen, Hilden, Germany) and cycle-sequenced using the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems). Sequencing was performed on an ABI PRISM 310
Table I. Oligonucleotide primers for reverse transcriptase-polymerase chain reaction analysis of the liver kinase B1 (LKB1) gene (GenBank accession number NM_000455).

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Amplicon size (bp)</th>
<th>Sequence</th>
<th>Primer location</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exons 1-3</td>
<td>585</td>
<td>F: 5'-AGT CGG AAC ACA AGG AAG GAC-3'</td>
<td>1041-1061</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: 5'-CTG GCT ATG CAG GTA CTC CAG-3'</td>
<td>1605-1625</td>
</tr>
<tr>
<td>Exons 4-7</td>
<td>502</td>
<td>F: 5'-GAG CTT TTC CCA GTG TG-3'</td>
<td>1548-1567</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: 5'-GGT CAG GAT GAT GTI T-3'</td>
<td>2021-2049</td>
</tr>
<tr>
<td>Exons 8-9</td>
<td>519</td>
<td>F: 5'-GAA AGG GAT GCT TGA GTA CGA A-3'</td>
<td>1973-1994</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: 5'-ACC CCA GAA GAC GAC T-3'</td>
<td>2473-2491</td>
</tr>
</tbody>
</table>

F: Forward primer; R: reverse primer.

sequence apparatus (Applied Biosystems). Specimens with LKB1 mutation were further confirmed with DNA samples.

Analysis of LKB1 Phe354Leu polymorphism. To detect the presence of LKB1 Phe354Leu polymorphism, genomic DNA was used for LKB1 exon 8 amplification with primers as follows: forward: 5'-GAG CTG GGT CGG AAA ACT G-3' and reverse: 5'-AGA AGC TGT CCT TGT TGC AGA-3'. PCR was carried out in a 25-μl final volume containing approximately 100 ng genomic DNA, 200 nM of each primer, 200 μM dNTPs, 1.5mM MgCl2, 1.25 U GoTaq® Flexi DNA Polymerase (Promega), and supplied buffer. PCR amplification consisted of initial denaturation at 95°C for 5 min followed by 35 cycles of 95°C for 30 sec, 62°C for 30 sec, and 72°C for 30 sec prior to a final elongation process at 72°C for 5 min. Sequence analysis was performed as for analysis using cDNA samples.

Statistical analysis. All statistical analyses were performed using SPSS software package, version 14 (SPSS, Chicago, IL, USA). Overall survival probabilities were calculated by Kaplan–Meier method, and differences in survival distribution were compared by the log-rank test. Overall survival was calculated from the date of first diagnosis to the date of last follow-up or death from any cause. Values of p<0.05 were considered statistically significant.

Results

Patient population. A total of 85 patients with de novo AML were included in this study (49 men and 36 women), aged 21-86 years (median age=52.3 years). In the entire patient population, the complete remission rate was 51.8% and the mean overall survival was 648 days. Molecular markers were also analyzed for all available diagnostic bone marrow samples. Mutant NPM1 was observed in 37 out of 85 patients (43.5%), FLT3-ITD in 20/85 (23.5%), FLT3-TKD in 7/85 (8.2%), mutant CEBPA in 32/85 (37.6%), mutant IDH1 in 3/85 (3.5%) and mutant IDH2 in 11/85 (12.9%). At least one molecular maker mutation was identified in 69/85 (81%) patients. Mutant FLT3 was more frequently associated with the presence of mutant NPM1 (p<0.001). The presence of mutation of FLT3 led to significantly worse overall survival (p<0.01). Clinical characteristics and the frequencies of the molecular marker of the 85 patients with de novo CN AML at the time of the initial diagnostic evaluation are summarized in Table II.

LKB1 gene mutations in patients with de novo CN AML. Here we reported our results about the mutation status of LKB1 in patients with de novo CN AML. One silent mutation (837C>T) of LKB1 was detected in a 22-year-old male patient who also had CEBPA mutation. This is in agreement with previous reports that LKB1 mutations were relatively rare in patients with cancer who did not have Peutz-Jeghers syndrome, except for non-small cell lung cancers (NSCLCs) (18, 19). In addition, another alteration, Phe to Leu at codon 354 (Phe354Leu), was detected in 7% (6 out of 85) of our patients with AML (Figure 1). Phe354Leu was reported to be a rare polymorphism, the same mutation has been found in Koreans with left-sided colorectal cancer (in 6.3%) as well as in cancer-free controls (in 5.6%) from the same population (33). This mutation was found in one Peutz-Jeghers syndrome family including many affected relatives and the change seems to co-segregate with the disease (34).

Clinical characteristics and outcome of patients with AML with LKB1 Phe354Leu polymorphism. In this study, we found all six patients with LKB1 Phe354Leu polymorphism achieved complete remission after treatment. Among the six patients with Phe354Leu polymorphism, four of them were diagnosed at 31-36 years of age which was younger than the average age of whole patient group at diagnosis (52.3 years). Except one patient who had long overall survival (1,786 days), the other five patients had an average overall survival of 305 days (range=106-452 days), which is shorter than the overall survival of patients with AML overall (648 days). Concurrent mutations of other molecular markers, NPM1, FLT3, and CEBPA, were detected in all patients with LKB1 Phe354Leu polymorphism. Three patients with LKB1 Phe354Leu polymorphism.
Phe354Leu polymorphism had NPM1 mutation, three patients had FLT3 mutation and four patients had CEBPA mutation. None of the patients had IDH1 and IDH2 mutations. Compared to the overall survival of patients with NPM1 mutation only (613 days), the overall survival of the three patients with both LKB1 Phe354Leu polymorphism and NPM1 mutation was shorter (322 days). Compared to the overall survival of patients with FLT3 mutation only (446 days), the overall survival of the three patients with both LKB1 Phe354Leu polymorphism and FLT3 mutation was also shorter (186, 106 and 377 days, respectively). Except for one patient with both LKB1 Phe354Leu polymorphism and CEBPA mutation who was diagnosed at older age (69 years) and had longer overall survival (1,786 days), the overall survival of the other three patients with both LKB1 Phe354Leu polymorphism and CEBPA mutation (411 days) was shorter than the overall survival of the patients carrying only the CEBPA mutation (590 days). The clinical characteristics of the patients carrying the LKB1 Phe354Leu polymorphism are listed in Table III.

Discussion

It has long been known that tumor cells undertake aerobic glycolysis, the so-called Warburg effect. The alteration of the function of metabolic enzymes might help resolve the

<table>
<thead>
<tr>
<th>NPM1</th>
<th>FLT3</th>
<th>CEBPA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>Mutant</td>
<td>Wild-type</td>
</tr>
<tr>
<td>All patients</td>
<td>48 (56.5%)</td>
<td>37 (43.5%)</td>
</tr>
<tr>
<td>Age at diagnosis (range)</td>
<td>52.3 (21-86)</td>
<td>49.5 (21-86)</td>
</tr>
<tr>
<td>&lt;60 Years old, n</td>
<td>48</td>
<td>29</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(male/female)</td>
<td>49/36</td>
<td>29/19</td>
</tr>
<tr>
<td>WBC count, x10^9/l (range)</td>
<td>57.0 (0.1-328.3)</td>
<td>36.6 (0.3-219.9)</td>
</tr>
<tr>
<td>Bone marrow blasts, % (range)</td>
<td>56.1 (4-95)</td>
<td>55.0 (4-94.2)</td>
</tr>
<tr>
<td>Hemoglobin, g/dl (range)</td>
<td>8.3 (3.5-15.4)</td>
<td>8.2 (3.5-13.1)</td>
</tr>
<tr>
<td>Platelet count, x10^9/l (range)</td>
<td>78.5 (3-908)</td>
<td>68.3 (3-90.8)</td>
</tr>
<tr>
<td>Hemoglobin, % (range)</td>
<td>56.1 (4-95)</td>
<td>55.0 (4-94.2)</td>
</tr>
<tr>
<td>Platelet count, % (range)</td>
<td>56.1 (4-95)</td>
<td>55.0 (4-94.2)</td>
</tr>
<tr>
<td>Overall survival, days (range)</td>
<td>648</td>
<td>674 (4-3161)</td>
</tr>
</tbody>
</table>

CEBPA: CCAAT/enhancer binding protein alpha; FLT3: fms-related tyrosine kinase 3; IDH1/2: isocitrate dehydrogenase [nicotinamide adenine dinucleotide phosphate (+)]1/2; NPM1: nucleophosmin 1; WBC: white blood cell.

Table II. Clinical characteristics of patients with cytogenetically normal acute myeloid leukemia.
Figure 1. Sequence analysis of liver kinase B1 (LKB1) mutation. Reverse transcriptase-polymerase chain reaction amplification of cDNA and DNA from patients with acute myeloid leukemia revealed the 1062C>G mutation, which leads to Phe354Leu change in the protein.

Table III. The clinical characteristic of patients with acute myeloid leukemia with liver kinase B1 (LKB1) Phe354Leu polymorphism.

<table>
<thead>
<tr>
<th>Patient number</th>
<th>Gender</th>
<th>Age at diagnosis (years)</th>
<th>FAB classification</th>
<th>Survival (days)/status</th>
<th>WBC count (×10⁹/l)</th>
<th>Bone marrow blast (%)</th>
<th>Hemoglobin (g/dl)</th>
<th>Platelet count (×10⁹/l)</th>
<th>Other molecular markers</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Male</td>
<td>69</td>
<td>M2</td>
<td>1786/alive</td>
<td>29.56</td>
<td>35.2</td>
<td>8.7</td>
<td>4</td>
<td>CEBPA mutation</td>
</tr>
<tr>
<td>2</td>
<td>Male</td>
<td>33</td>
<td>M4</td>
<td>186/dead</td>
<td>64.76</td>
<td>69.4</td>
<td>9.9</td>
<td>58</td>
<td>NPM1, FLT3 mutation</td>
</tr>
<tr>
<td>3</td>
<td>Male</td>
<td>54</td>
<td>M2</td>
<td>106/dead</td>
<td>43.31</td>
<td>93.5</td>
<td>5.3</td>
<td>20</td>
<td>FLT3 mutation</td>
</tr>
<tr>
<td>4</td>
<td>Female</td>
<td>36</td>
<td>M1</td>
<td>452/dead</td>
<td>39.00</td>
<td>51.1</td>
<td>8.8</td>
<td>4</td>
<td>CEBPA mutation</td>
</tr>
<tr>
<td>5</td>
<td>Female</td>
<td>34</td>
<td>M4</td>
<td>403/dead</td>
<td>15.58</td>
<td>29.5</td>
<td>6.6</td>
<td>62</td>
<td>NPM1, CEBPA mutation</td>
</tr>
<tr>
<td>6</td>
<td>Female</td>
<td>31</td>
<td>M4</td>
<td>377/dead</td>
<td>100.71</td>
<td>8.3</td>
<td>7.7</td>
<td>120</td>
<td>NPM1, FLT3, CEBPA mutation</td>
</tr>
</tbody>
</table>

FAB: French-American-British classification; CEBPA: CCAAT/enhancer binding protein alpha; FLT3: fms-related tyrosine kinase 3; NPM1: nucleophosmin 1; WBC: white blood cell.
enigmatic, aerobic glycolytic state of cancer cells (35). For example, two metabolism-related genes, \emph{IDH1} and \emph{IDH2}, are frequently mutated in different cancer types including CN AML (36, 37). Recently, the molecular characterization of the LKB1/AMPK signaling pathway as a tumor-suppressor axis further supports the link between cancer and metabolism (16). Studies on HSC and leukemia cells have also emphasized the potential value of LKB1/AMPK modulation in hematological malignancies (22-26).

Here we reported our results on the mutation status of \emph{LKB1} in patients with de novo CN AML. We only found one silent mutation (837C>T) in our AML specimens. This is in agreement with previous reports that \emph{LKB1} gene mutations were found to be relatively rare in cancer from patients without Peutz-Jeghers syndrome except for non-small cell lung cancer (NSCLC) (18, 19). In addition, previous reports have suggested the \emph{LKB1} mutations were infrequent in patients of Asian origin with NSCLC (3%) compared to those found in NSCLC tumors and cell lines derived from patients of Caucasian origin (30%) (32, 38). The difference in \emph{LKB1} mutation frequencies between these two populations might be related to cigarette smoking history. These observations also indicate the possibility that \emph{LKB1} alterations might be induced by ethnic and lifestyle or environmental factors (32, 39).

\emph{LKB1} Phe354Leu polymorphism was observed in 7% (6 out of 85) of our CN-AML patients. This polymorphism occurs in the C-terminal region of LKB1 rather than in the kinase domain. In a study by Forcet et al., the Phe354Leu alteration lessened LKB1-mediated activation of the AMPK and impaired downstream signaling, and diminish LKB1 ability to maintain the polarity of cells (40). Moreover, this mutation was found in one Peutz-Jeghers syndrome family including many affected relatives and the change seems to co-segregate with the disease (34). Results of these studies suggested Phe354Leu alteration is associated with cancer predisposition. In our study, the patients with AML with \emph{LKB1} Phe354Leu polymorphism were diagnosed at younger ages and had worse overall survival. \emph{LKB1} Phe354Leu polymorphism also occurred concurrently with \emph{NPM1}, \emph{FLT3}, and \emph{CEBPA} mutations. The concurrent \emph{LKB1} Phe354Leu polymorphism in patients with CN-AML seems to have a worse impact on the overall survival.

Our results indicate that \emph{LKB1} Phe354Leu polymorphism may play an important role in leukemogenesis and represents a poor prognostic factor. Additional studies are needed to clarify the clinical implication of \emph{LKB1} mutations in leukemia and whether \emph{LKB1} mutations occur concurrently with other molecular makers and have mutual impact on prognosis.

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