Downregulation of LKB1 suppresses Stat3 activity to promote the proliferation of esophageal carcinoma cells

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Received May 22, 2013; Accepted January 14, 2014

DOI: 10.3892/mmr.2014.2071

Abstract. The tumor suppressor liver kinase B1 (LKB1) encodes a serine/threonine kinase. The defect in LKB1 is the primary cause of Peutz-Jeghers syndrome (PJS). Inactivation of LKB1 by mutations or loss of LKB1 expression is associated with ovarian, lung and pancreatic cancer; however, the correlation between LKB1 and esophageal carcinoma remains unknown. Thus, quantitative PCR was performed to determine the clinical significance of LKB1 expression in 60 cases of esophageal cancer and its adjacent normal epithelium. LKB1 expression was observed to significantly downregulate the accompanying cancer progression, which was verified at the protein level by western blot analysis. Furthermore, the phosphorylated signal transducer and activator of transcription 3 (Stat3) level is reversibly associated with LKB1 expression. To determine the function of LKB1 in esophageal cancer, LKB1 expression is induced in TE1 esophageal cancer cells. The results show that LKB1 overexpression suppresses the proliferation of TE1 cells, downregulates the expression of cyclin D1 and Myc and represses Stat3 phosphorylation. Suppression of cell proliferation and cyclin D1 expression by LKB1 is fully inhibited by constitutively active Stat3C coexpression, suggesting that LKB1 inhibits esophageal cancer cell proliferation through suppression of Stat3 transaction. In conclusion, downregulation of LKB1 expression suppresses Stat3 activity that may promote tumor growth during esophageal cancer progression.

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Key words: liver kinase B1, Peutz-Jeghers syndrome, signal transducer and activator of transcription 3, esophageal carcinoma cells

Introduction

Esophageal carcinoma is one of the most common types of malignancy, with highest mortality rate worldwide (1). Previous studies, which have led to advances in the diagnosis, staging and treatment of esophageal carcinoma have improved patient survival (2,3). However, effective approaches to treat esophageal cancer remain elusive due to the incomplete understanding of the molecular mechanisms of esophageal cancer. Therefore, it is crucial to investigate the mechanisms underlying esophageal cancer incidence and progression.

The serine/threonine kinase liver kinase B1 (LKB1) is a known tumor suppressor responsible for the inherited human cancer disorder Peutz-Jeghers syndrome (PJS) (4). Previous studies demonstrated that LKB1 mutations or abnormal expression of LKB1 are also associated with lung, colorectal, testis, ovarian and pancreatic cancer (5-8). LKB1 inhibits tumor cell cycle progression by inducing p21 and p53 gene expression dependent on its kinase activity (9,10). LKB1 deficiency leads to the induction of matrix metaloproteinase-2 (MMP2), MMP9 and vascular endothelial growth factor (VEGF) (11,12). Notably, these genes are also regulated by signal transducer and activator of transcription 3 (Stat3) activity in cell growth and tumorigenesis (13-16). Stat3 has been identified as an oncogene that is frequently activated in various cancer cells, including esophageal cancer (12,17-19). However, the biological functions of LKB1 in esophageal carcinoma have not been elucidated. Although it has been reported that LKB1 inhibits activation of Stat3 in papillary thyroid carcinoma (12), the association between LKB1 expression and Stat3 activity in esophageal cancer remains unclear.

In the current study, esophageal carcinoma and adjacent non-cancer tissue were collected from 60 patients for quantitative polymerase chain reaction (qPCR) analysis. LKB1 expression was observed to be significantly lower in esophageal carcinoma tissue compared with the adjacent normal epithelium, correlating with tumor node metastasis (TNM) stages. The *in vitro* study using the esophageal carcinoma cell line, TE1, revealed that LKB1 overexpression inhibits TE1 cell proliferation and impairs the characteristics of cancer cells. Furthermore, LKB1 functions as a tumor suppressor to downregulate cyclin D1 expression through inhibition of Stat3 activity. These findings indicate that downregulation of LKB1 expression may derepress Stat3 activity to promote esophageal carcinoma genesis, which provides an important theoretical basis for diagnosis and treatment of esophageal cancer.

Materials and methods

Samples and cell lines. A total of 60 surgically resected esophageal carcinoma specimens and adjacent normal epithelium were collected at the General Hospital of the People's Liberation Army (Beijing, China) from January 2009 to December 2011. Tumors were classified histologically using the Guide Lines for the Clinical and Pathologic Studies on Carcinoma of the Esophagus (20). There were five stage I; 24 stage II; 19 stage III; and 12 stage IV esophageal cancer patients and their mean age was 57.5 years (41-72 years). The conditions of patients are summarized in Table I. All samples were individually fresh-frozen in a -80°C refrigerator. Equal weight of cancer or normal samples were pooled together, respectively, according to different phases for western blot analysis, and each sample was individually subjected to RNA extraction and qPCR evaluation. Informed consent was obtained from each patient prior to surgery. The study was approved by the General Hospital of the People's Liberation Army Clinical Research Ethics Committee. Survival was calculated from the date of surgery to the date of the latest follow-up visit or mortality due to recurrent esophageal cancer. TE1 cells were originally obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA) and cultured according to ATCC guidelines.

RNA extraction and qPCR. Total RNA was isolated from tissue samples with TRIzol Reagent (Life Technologies, Carlsbad, CA, USA), following the manufacturer's recommendations. Total RNA (2.5 mg) was reverse transcribed using Superscript III Reverse Transcriptase (Invitrogen Life Technologies, Carlsbad, CA, USA) and amplified with SYBR-Green Real-time PCR Master mix (Toyobo, Osaka, Japan). The primers (Sangon Biotech, Shanghai, China) used for amplification of encoding genes were as follows: Forward: 5'-CGAAGTCAACGGATTTGG TCGTAT-3' and reverse: 5'-AGCCTTCTCGGTGGTGAA GAC-3' for glyceraldehyde 3-phosphate dehydrogenase (GAPDH); forward: 5'-GAGCTGATGTCGGTGGGTATG-3' and reverse: 5'-CACCTTGCCGTAAGAGCCT-3 for LKB1; forward: 5'-GCTGCGAAGTGGAAACCATC-3' and reverse: 5'-CCTCCTTCTGCACACATTTGAA-3' for cyclin D1; forward: 5'-TACCCTCTCAACGACAGCAG-3' and reverse: 5'-TCTTGACATTCTCCTCGGTG-3' for Myc; forward: 5'-TGTCCGTCAGAACCCATG-3' and reverse: 5'-TGGGAAGGTAGAGCTTGG-3' for p21; and forward: 5'-TAGTATTGTATTAGGTAGGGGGCGC-3' and reverse: 5'-TATCGATAACCCGAAAAACGTT-3' for p16.

Western blot analysis. The tissues or cells were harvested in lysis buffer containing 150 mM NaCl, 50 mM Tris-HCl (pH 8.0), 0.1% SDS, 0.5% sodium deoxycholate, 5 mM EDTA, 1% Nonidet P-40, 0.25 mM phenylmethanesulfonyl fluoride and protease inhibitors. The lysates were separated by SDS-PAGE and then proteins were transferred to polyvinylidene fluoride membranes (Millipore, Bedford, MA, USA), followed by blocking in 5% skimmed milk and

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Phase	N (%)	Male/female	2-YS rate (%)
I	5 (8.3)	2/3	100
II	24 (40.0)	14/10	70.8
III	19 (31.7)	10/9	42.1
IV	12 (20.0)	7/5	0

2-YS, 2-year survival rate.

immunoblotting with anti-LKB1 mouse monoclonal antibody (1:500), anti-Stat3 rabbit polyclonal antibody (1:1,000) (both from Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), anti-pY705-Stat3 rabbit monoclonal antibody (p-Stat3) (1:1,000; Cell Signaling Technology, Danvers, MA, USA) and anti-GAPDH rabbit polyclonal antibody (1:10,000; Abcam, Cambridge, MA, USA).

Transient transfection and reagents. TE1 cells were transfected using Lipofectamine 2000 reagents (Life Technologies) following the manufacturer's instructions, when the cells reached 60-70% confluency. Stat3C, a constitutively active form of Stat3, was purchased from Addgene plasmid repository (Cambridge MA, USA). Stat3 inhibitor Stattic was purchased from Calbiochem (La Jolla, CA, USA).

Cell proliferation assay. Cell proliferation was detected by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as previously described (21). Optical density (OD)570 values were determined from cultured TE1 cells at days 0-4. The mean values of three wells were calculated as the final OD value. Cell proliferation was considered to be in linear proportion to the color measurements.

Statistical analysis. Data are expressed as mean counts \pm standard error of the mean. All results were obtained from three independent experiments for analysis. The Statistical Package for the Social Sciences (SPSS; SPSS Inc., Chicago, IL, USA) was used to analyze data. Student's t-test was analyzed and P<0.05 was considered to indicate a statistically significant difference.

Results

Downregulation of LKB1 expression during esophageal carcinoma progression. Previously, defects of LKB1 were reported to be associated with lung, ovarian and pancreatic cancer (5,6). To evaluate the association between LKB1 expression and esophageal carcinoma, 60 esophageal cancer patients at phases I-IV were collected for qPCR and 2-year survival rate analysis. The 2-year survival rates at phase I-IV were 100, 70.8, 42.1 and 0, respectively (Table I), suggesting that the 2-year survival rate was highly associated with esophageal carcinoma progression. Therefore, it is valuable to investigate the mechanism of esophageal cancer during the early stage. The LKB1 mRNA levels in 60 specimens were examined by qPCR and the results show that LKB1 expression was significant in



Figure 1. LKB1 expression is downregulated during esophageal carcinoma progression. The specimens from five stage I; 24 stage II; 19 stage III and 12 stage IV patients were subjected to qPCR analysis individually with human LKB1 specific primers. The samples from the adjacent normal tissues were set as con. LKB1, liver kinase B1; qPCR, quantitative PCR; con, control.



Figure 2. LKB1 expression is reversely correlated with Stat3 activity in esophageal carcinoma. An equal weight of cells from each cancer tissue or adjacent normal epithelium (con) was resected and samples with the same phases were combined for lysis and western blot analysis. The protein levels of LKB1, phosphoryleted (p)-Stat3, Stat3 and GAPDH were determined in phase I-IV specimens and adjacent normal tissues. LKB1, liver kinase B1; Stat3, signal transducer and activator of transcription 3; con, control; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

cancer tissues compared with the adjacent normal epithelium. Furthermore, LKB1 expression was gradually decreased from phase I to IV (Fig. 1). This demonstrates that progressive LKB1 downregulation may be associated with esophageal cancer development.

Revisable association between LKB1 expression and Stat3 activity in esophageal carcinoma. To verify the protein level change of LKB1 in esophageal carcinoma, the tissues from the same phases were pooled together for western blot analysis. LKB1 protein level was observed to be markedly lower in cancer tissues (phase I-IV) compared with the adjacent normal epithelium (Fig. 2), which is similar with the mRNA level change (Fig. 1). It was hypothesized that Stat3 was activated by interleukin (IL)-6 in esophageal cancer (19) and that the same genes are regulated by Stat3 activation and LKB1 deficiency (11,12,16). To investigate the association between LKB1

expression and Stat3 activity, the phosphorylated Stat3 level was also examined in the present study. Notably, Stat3 phosphorylation was detected in cancer tissues from phase I-IV, while it was maintained at an extremely low level in the adjacent normal tissue, and Stat3 expression was not altered in the cancer and normal specimens (Fig. 2). Thus, LKB1 downregulation was associated with Stat3 phosphorylation in esophageal cancer.

LKB1 overexpression inhibits the proliferation of esophageal carcinoma cells and represses Stat3 activity. The tumor suppressor LKB1 led to cell growth in PJS (9,10). To investigate whether LKB1 functions as a tumor suppressor in esophageal carcinoma, the TE1 esophageal cancer cell line was applied for functional investigation. Since LKB1 expression was extremely low in TE1 cells, LKB1 was overexpressed to examine the cell proliferation rate by MTT assays in TE1 cells. LKB1 overexpression inhibited cell growth (Fig. 3A). Notably, the characteristics of cancer cells were impaired by LKB1. The expression of tumor suppressor genes p16 and p21 was upregulated and the mRNA level of cyclin D1 and Myc was downregulated (Fig. 3B), suggesting that LKB1 may also result in cell growth arrest in esophageal carcinoma to inhibit tumor growth.

Based on LKB1 expression level and Stat3 activation change (Fig. 2), it was investigated whether Stat3 activity was modulated by LKB1. The phosphorylated Stat3 level was determined following LKB1 overexpression and the results show that Stat3 phosphorylation was suppressed by LKB1 (Fig. 3C). This is consistent with the expression patterns in normal epithelial cells with high LKB1 and low Stat3 phosphorylation levels (Fig. 2).

Constitutively active Stat3 activity blocks the LKB1-elicited suppression of proliferation in esophageal carcinoma cells. As LKB1 suppresses cell proliferation of TE1 cells and inhibits Stat3 phosphorylation, it was investigated whether LKB1 functions through downregulating Stat3 activity. A chemical Stat3 inhibitor, Stattic, was used to block Stat3 transactivity and it was observed that Stattic significantly inhibited cell proliferation, similar to that of the LKB1 overexpression effects (Fig. 4A). By contrast, overexpression of a constitutively active Stat3 form, Stat3C, promoted TE1 cell growth. When LKB1 and Stat3C were co-transfected into TE1 cells, LKB1-elicited suppression of cell proliferation was fully inhibited by forced activation of Stat3 (Fig. 4A). The expression of cyclin D1 under the same conditions was also investigated. Cyclin D1 expression was observed to be downregulated by LKB1 overexpression, and Stattic and Stat3C markedly upregulated the cyclin D1 transcriptional level. Furthermore, a decrease in LKB1-induced cyclin D1 was completely rescued by constitutively active Stat3C. Thus, LKB1 suppresses TE1 cell proliferation by inhibiting Stat3 activity and further downregulating cyclin D1 gene expression.

Discussion

The tumor suppressor LKB1 has intrinsic serine/threonine kinase activity and its defects cause PJS and various cancers (4-6,22,23). It is unknown whether LKB1 is associated



Figure 3. LKB1 OV inhibits the proliferation of esophageal carcinoma cells and represses Stat3 activity. (A) LKB1 was overexpressed in TE1 cells by transfection of pcDNA3-LKB1 or pcDNA3 vector as a con. MTT assays were performed on the indicated days with OD570 evaluation. (B) Control and LKB1 overexpressing TE1 cells were collected at day four for mRNA expression analysis and the expression of LKB1, p16, p21, cyclin D1 and Myc were examined by qPCR. (C) Western blot analysis of LKB1, phosphorylated (p)-Stat3, Stat3 and GAPDH levels in control and LKB1-overexpressing TE1 cells. *P<0.05, compared with the control. LKB1, liver kinase B1; OV, overexpression; OD, optical density; Stat3, signal transducer and activator of transcription 3; con, control; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; qPCR, quantitative PCR; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.



Figure 4. Constitutive Stat3 activity blocks LKB1-elicited suppression of proliferation in esophageal carcinoma cells. (A) Constitutively active Stat3 form Stat3C was transfected alone or co-transfected with LKB1 into TE1 cells. Empty vectors served as con. TE1 cells were supplemented with Stattic (10 μ M) or DMSO. MTT assays were performed to evaluate cell proliferation under indicated conditions following four days of culture. (B) RNA samples were collected under the same conditions in (A) and the cells were subjected to qPCR analysis of cyclin D1 expression. *P<0.05. Stat3, signal transducer and activator of transcription 3; LKB1, liver kinase B1; OV, overexpression; OD, optical density; DMSO, dimethylsulfoxide; con, control; MTT, 3-(4,5-dimethylthi-azol-2-yl)-2,5-diphenyltetrazolium bromide; qPCR, quantitative PCR; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

with the incidence of esophageal carcinoma. In the current study, qPCR analysis of 60 esophageal cancer specimens revealed that LKB1 mRNA and protein level is progressively downregulated during the progression of esophageal cancer compared with the adjacent normal epithelium. This observation indicated that downregulation of LKB1 in the esophageal epithelium may be applied as an early diagnostic marker in the clinical process.

As a tumor suppressor, LKB1 downregulation may cause normal cells to lose their ability to suppress tumorigenesis, which has been observed in other types of tumors (9,10). Therefore, LKB1 was hypothesized to be capable of suppressing the esophageal carcinomagenesis. To verify this hypothesis, LKB1 was overexpressed in the TE1 esophageal carcinoma cell line cells *in vitro*. LKB1 overexpression also resulted in cell growth inhibition in TE1 cells, which is consistent with previous studies (9,10). The gene expression signature of LKB1-overexpressing TE1 cells includes p16 and p21 elevation, as well as cyclin D1 and Myc downregulation. It demonstrates that LKB1 may suppress cell growth dependent on modulating cell cycle arrest, as in other types of tumor cells (10,12).

Stat3 is a well-known oncogene that is activated in multiple types of cancers, including esophageal carcinoma (17-19). A previous study reported that Stat3 transactivity was suppressed by LKB1 (12), while the association between Stat3 activity and LKB1 in esophageal cancer remains unclear. Generally, Stat3 phosphorylation levels represent Stat3 activity (24), thus, p-Stat3 level was also determined in phase I-IV patients. Stat3 phosphorylation was observed to be downregulated in cancer tissue with low p-Stat3 levels, which is negatively correlated with high LKB1 expression. LKB1 overexpression repressed Stat3 phosphorylation in TE1 cells when LKB1 inhibited cell proliferation. Another tumor suppressor, p53, was also reported to regulate Stat3 phosphorylation and DNA binding activity in prostate cancer cells (25). This suggests that it is possible for tumor suppressors to inhibit Stat3 transcriptional activity.

Stat3 activity inhibition and LKB1 overexpression showed inhibitory effects on TE1 cell growth, while constitutively active Stat3 promoted cell proliferation. Notably, LKB1-elicited suppression of cell growth was fully inhibited by forced activation of Stat3, demonstrating that LKB1 functions in cell proliferation through modulation of Stat3 phosphorylation. This is observed in the functional correlation between LKB1 and Stat3 activity in esophageal carcinoma cells. A previous study proposed that LKB1 reduces the binding of Stat3 to cyclin D1 and the VEGF promoter to inhibit the target gene expression (12). This is consistent with the observations of the present study that LKB1 downregulated cyclin D1 expression through suppression of Stat3 activity. The results of these studies demonstrate that LKB1-mediated suppression of Stat3 target genes is dependent on the phosphorylation status of Stat3.

In conclusion, the current study revealed that the downregulation of LKB1 expression is associated with esophageal carcinoma progression in 60 cases. The esophageal cancer development was also accompanied with an increase in the p-Stat3 level. Functional investigation in TE1 cells showed that suppression of esophageal cancer cell growth and cyclin D1 expression by LKB is dependent on inhibition of Stat3 activity. Thus, LKB1 is hypothesized to act as a tumor suppressor to inhibit cell growth through counteracting oncogenic Stat3 activity and that downregulation of LKB1 may derepress its inhibition on Stat3 activation to promote esophageal carcinomagenesis. The present study may provide a potential mechanism underlying the initiation of esophageal carcinomagenesis and it provides information for the exploration of the latent therapy target for esophageal cancer.

Acknowledgements

The study was supported by Wu Jieping medical fund: The animal experimental study on ¹¹C-gefitinib therapy of esophageal carcinoma (number: 320.6799.1141).

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