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Liver kinase B1 expression (LKB1) is repressed by estrogen receptor alpha (ERα) in MCF-7 human breast cancer cells

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ABSTRACT

Background: Liver kinase 1 (LKB1) is emerging as a multifunctional protein, acting as a key metabolic enzyme, regulator of cell polarity, and transcription factor. Altered LKB1 expression has been linked with various cancers and may be a potential prognostic marker. While the functional role of LKB1 continues to undergo intensive investigation, the molecular mechanisms that regulate its expression remain to be defined more clearly. Recent reports have established a possible link between estrogen receptor alpha (ERα) signaling and LKB1 in MCF-7 human breast cancer cells. The current study aimed to investigate whether LKB1 is transcriptionally regulated by ERα in MCF-7 cells.

Methods: siRNA transfections were used to transiently knock down LKB1 and ERα. LKB1 and ERα mRNA and protein levels were evaluated by real-time PCR and Western blotting, respectively. An approximately 3 kilobase pair human LKB1 promoter construct and various truncations were generated, transfected into MCF-7 cells, and luciferase reporter assays were performed. Cells were also treated with various doses of 17-β-estradiol (E2) to evaluate the effect on LKB1 and ERα mRNA levels.

Results: LKB1 mRNA and protein levels were significantly lower in ERα-positive MCF-7 compared to ERα-negative MDA-MB-231 breast cancer cells, suggesting that ERα may act as a repressor. siRNA-mediated knock-down of ERα in MCF-7 cells significantly increased LKB1 promoter activity and expression at the mRNA and protein levels, and computational analysis revealed the presence of several putative estrogen response element (ERE) DNA binding sites in the LKB1 promoter region. In addition, treatment with E2 led to an increase in LKB1 expression, concomitant with decreased expression of ERα in MCF-7 cells. The E2-mediated increase was abrogated by pretreatment with actinomycin D, supporting that the observed changes in LKB1 levels were transcriptionally regulated.

Conclusions: ERα repressively modulates the expression of LKB1 at the transcriptional level. Targeting the expression of LKB1 by modulating ERα signaling may provide a potential approach to further evaluate its function in ERα-positive breast cancers.

1. Introduction

The serine/threonine kinase LKB1, a putative tumor suppressor, has also been linked to cell polarity and metabolism, acting as an upstream phosphorylator of AMPK, a key regulator of cellular energy homeostasis (reviewed in [1]). Recently, the LKB1-AMPK pathway has been shown to inhibit aromatase expression in human breast stromal cells [2], and metformin-mediated activation of this pathway in human breast adipose stromal cells inhibits aromatase expression [3]. While numerous studies have examined the function of LKB1 in cancer, and mutational analyses within the coding sequence of the LKB1 gene have been performed, relatively little is known regarding how LKB1 expression itself is regulated. It has been suggested that epigenetic mechanisms alter the expression of LKB1 in various cancers [4,5]. The LKB1 promoter was reported to be hypermethylated in 45% of papillary breast carcinomas that arise sporadically and are related to Peutz-Jeghers syndrome, while in breast cell lines and primary tumors, the promoter was primarily unmethylated, similar to the pattern observed in normal cells and tissues [4]. This suggests that activation/inactivation of the LKB1 promoter is also driven by other mechanisms, in a manner depending on the cancer subtype. Metformin, which has gained considerable attention not only as an anti-diabetic drug, but also as a potential anti-cancer agent, mildly upregulates the level of total LKB1 protein, also modestly increasing LKB1 promoter activity in transfected COS-7 cells [3]. We have observed similar metformin-mediated effects on the LKB1 promoter in human MCF-7 breast cancer cells (unpublished results). A recent study reported that ERα binds to the LKB1 promoter and that LKB1 promoter activity and expression are inhibited by 17-β-estradiol (E2) in MCF-7 cells [6].

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Furthermore, it was recently shown that LKB1 contains a nuclear localization sequence, indicating that it may act as a transcription factor [7]. Interestingly, LKB1 was reported to bind to ERα in MCF-7 cells, functioning as a co-activator to up-regulate the activity of the cyclin D1 promoter without altering the expression of ERα itself [7]. In addition, LKB1 was shown to be recruited to the promoter of the c-myconcogene, which is known to be activated by ERα [7]. That LKB1 enhances the activity of ERα, which is associated with an increased risk of breast cancer, seems contradictive, given that LKB1 also acts as a tumor suppressor. However, others have provided evidence that tumor suppressors may function as nuclear receptor coactivators [8–10]. It has also been suggested that activation of the LKB1-AMPK signaling pathway, in response to specific factors that increase the ratio of AMP/ATP, may provide a survival mechanism and protect cells from apoptosis [11]. Therefore, the role of LKB1 in the onset and progression of breast cancer remains to be definitively established. Clearly, LKB1 is emerging as an important player in cancer biology, and gaining a better understanding of this multi-tasker at all levels could potentially lead to the development of novel therapies. Given emerging evidence supporting a relationship between LKB1 and ERα, our aim in the current investigation was to investigate how LKB1 expression is regulated in human ERα-positive MCF-7 breast cancer cells.

2. Methods

2.1. Policy and ethics

The work described in the current investigation has been carried out, where applicable, in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans (http://www.wma.net/en/30publications/10policies/b3/index.html), EU Directive 2010/63/EU for animal experiments (http://ec.europa.eu/environment/chemicals/lab_animals/legislation_en.html), and Uniform Requirements for manuscripts submitted to Biomedical journals (http://www.icmje.org).

2.2. Cloning

The human *LKB1* 5'UTR and proximal promoter region from −1889 to +1109 bp was cloned using MCF-7 genomic DNA. Pfx polymerase and Enhancer solution (Invitrogen) were applied in a PCR with the following primers: forward (FOR): 5′-GTACAAATTTTCGTATAGCTCATAAG-3′ and reverse (REV): 5′-CTCCAGGACCCTGGGTCCAGC-3′. The bi-directionally sequenced promoter fragment was subcloned into the pGL3-Basic firefly luciferase expression vector (Promega), resulting in pGL3-LKB1p (−1889/+1109). Five promoter truncations were constructed by PCR using the pGL3-LKB1p plasmid as template and bi-directionally sequenced. Forward primers with XhoI sites and a common reverse primer with a HindIII site facilitated transfer of products into pGL3-Basic, resulting in pGL3-Trunc1 (−1083/+1109), pGL3-Trunc2 (−436/+1109), pGL3-Trunc3 (+270/+1109), pGL3-Trunc4 (+696/+1109), and pGL3-Trunc5 (+923/+1109) deletion plasmids. Promoter response elements were computationally identified using software based on the Transfac database.

2.3. Cell culture, transient transfections, and reporter gene assays

MCF-7 and MDA-MB-231 human breast cancer cells (ATCC) were maintained in DMEM, High Glucose (Invitrogen) supplemented with 10% fetal bovine serum (FBS) at 37 °C in 5% CO_2. Cells were seeded into 6-well plates, incubated overnight, and co-transfected using the Lipofectamine 2000 reagent (Invitrogen) following the manufacturer's recommendations with the LKB1 promoter constructs and pRL-TK, a control vector encoding the renilla luciferase reporter gene. Reporter assays were performed using the Dual Luciferase Assay System (Promega). Firefly luciferase activity was normalized by renilla luciferase activity, and the fold increase relative to pGL3-Basic was calculated.

2.4. Drug treatments

Cells seeded into 6-well plates were cultured in phenol red-free, serum-free media for 48 h prior to the addition of E2 (Sigma) at 0, 1, 10, and 100 nM. E2 was administered for 6 h and cells were collected for subsequent analyses. In addition, cells were also cultured in phenol red-free media in the presence of 10% FBS for 48 h, and then treated with 0, 100, and 500 nM of E2 for 6 h. Cells were pretreated with 10 μg of actinomycin D for 1 h in media containing serum prior to the addition of E2 at 500 nM for 6 h.

2.5. siRNA transfections

Cells were seeded into 6-well plates and cultured for 4 h. Transient siRNA transfections were performed using Hiperfect reagent (Qiagen) and siRNAs specifically targeting LKB1 or ERα (Qiagen) following the manufacturer's recommendations with modifications [12]. After 24 h, the media was changed, and at 72 h post-transfection, cells were harvested to analyze knock-down efficiency. For reporter assays, cells were transfected with pGL3 vectors after a 48 h siRNA transfection and harvested after 24 h at 37 °C.

2.6. Reverse transcription (RT) and real time PCR

cDNA was prepared from MCF-7, MDA-MB-231, and 184B5 (normal breast epithelial) cells. RT reactions were performed using 500 ng of total RNA and SuperScript III (Invitrogen) following the manufacturer's protocol. Primers used to amplify human *LKB1, ERα,* and the RNA polymerase II (*RPII)* housekeeping gene were designed based on modifications to those listed in PrimerBank (http://pga.mgh.harvard.edu/primerbank/index.html). *LKB1*: FOR 5′-GAGCTGATGTCGGTGGGTATG-3′ and REV 5′-CACCTTGCCGTAAGAGCCT-3′, 144 bp; ERα: FOR 5′-GGTGCCCCTCTATGACCTG-3′ and REV 5′-CCTCCCCCGTGATGTAATACTT-3′, 164 bp; *RPII-1*: FOR 5′-GGGTGCTGAGTGAGAAGGAC-3′ and REV 5′-AGCCATCAAAGGAGATGACG-3′, 138 bp. Cycling conditions for real time PCR included: 95 °C-1 min, 40 cycles of 95 °C-10 s and 60 °C-25 s, and melt peak determination. Amplification efficiencies were determined for each primer pair and the integrity of products was verified by gel electrophoresis. Relative mRNA levels were calculated using the $2^{-[\Delta][\Delta]Ct}$ method [13], and results are presented as fold relative to indicated controls.

2.7. Western blot analysis

Lysates were prepared in lysis buffer (20 mM Tris–Cl pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, $+Na_2H_2P_2O_7$, $+Na_3VO_4$) with protease inhibitors (Roche). A total of 30–50 μg of protein were subjected to SDS–PAGE gel electrophoresis, and blots transferred onto PVDF membranes were incubated in anti-LKB1 (1:1000, Cell Signaling Technology) or anti-ERα (1:500, Santa Cruz Biotechnology) antibodies followed by incubation with anti-rabbit (1:3000, Cell Signaling Technology) or anti-mouse (1:2500, Santa Cruz Biotechnology) IgG horseradish peroxidase, respectively. Signals were detected using the ECL Plus Western Blotting Detection System (Amersham Biosciences) and exposure to film. Membranes were reprobed with anti-Actin antibody (1:1000, MP) and anti-mouse IgG horseradish peroxidase (1:8000). Densitometric analyses were performed using ImageJ software. Results