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Homozygous Deletion of the *STK11/LKB1* Locus and the Generation of Novel Fusion Transcripts in Cervical Cancer Cells

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Abstract

The *STK11/LKB1* gene encodes a ubiquitously expressed serine/threonine kinase that is mutated in multiple sporadic cancers including non-small cell lung carcinomas, pancreatic cancers, and melanomas. *LKB1* affects multiple cellular functions including cell growth, cell cycle progression, metabolism, cell polarity and migration. To date, only a limited number of studies have assessed the status of *LKB1* in cervical cancers. Herein, we investigate DNA methylation, DNA mutation, and transcription at the *LKB1* locus in cervical cancer cell lines. We identified homozygous deletions of 25–85kb in the HeLa and SiHa cell lines. Deletion breakpoint analysis in HeLa cells revealed that the deletion resulted from an Alu-recombination mediated deletion (ARMD) and generated a novel *LKB1* fusion transcript driven by an uncharacterized CpG island promoter located ~1kb upstream of *LKB1*. Although the homozygous deletion in SiHa cells removes the entire *LKB1* gene and portions of the neighboring genes *SBNO2* and *c19orf26*, this deletion also generates a fusion transcript driven by the *c19orf26* promoter and comprised of both *c19orf26* and *SBNO2* sequences. Further analyses of public gene expression and mutation databases suggest that *LKB1* and its neighboring genes are frequently dysregulated in primary cervical cancers. Thus, homozygous deletions affecting *LKB1* in cervical cancers may generate multiple fusion transcripts involving *LKB1*, *SBNO2*, and *c19orf26*.

Keywords

LKB1; *STK11*; *SBNO2*; *c19orf26*; homozygous deletion; Alu recombination-mediated deletion; ARMD; fusion transcript; cervical cancer

INTRODUCTION

Mutation of the *STK11/LKB1* (serine–threonine kinase 11/Liver kinase B1) gene is the causative genetic defect underlying the autosomal-dominant Peutz-Jeghers syndrome (PJS) [1,2]. PJS is characterized by non-neoplastic hamartomatous polyps of the gastrointestinal tract

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and hyperpigmented macules on the lips and oral mucosa [3]. PJS patients also exhibit an elevated risk of developing certain cancers including those of the gastrointestinal tract, cervix, lung, breast, thyroid, prostate, and pancreas [3]. This cancer predisposition of PJS patients and recent research into the function of LKB1 support a role for *LKB1* as a tumor suppressor gene.

LKB1 encodes a ubiquitously expressed serine/threonine kinase that affects cell growth, cell cycle progression, glucose and fatty acid metabolism, mitochondrial function, cell polarity, migration, and angiogenesis [2,4,5]. By itself LKB1 is localized in the nucleus, however, upon interaction with its binding partners STRAD (STE20-related adaptor) and MO25 (mouse protein 25), LKB1 is exported into the cytoplasm as a fully active kinase complex [4,6]. The primary targets of LKB1 appear to be a family of AMP-activated protein kinases (AMPKs) [7]. AMPKs are serine/threonine kinases that are critical for the regulation of cellular metabolism pathways during energy deprivation such as starvation, ischemia, and hypoxia [8,9]. Additionally, the AMPK-related microtubule affinity regulating kinases (MARKs) are important LKB1 substrates that control cell polarity through their effects on tubulin dynamics [10]. However, non-AMPK-related targets likely also contribute to LKB1 function. For example, LKB1 interacts with and phosphorylates PTEN *in vitro*, a dual specificity phosphatase that antagonizes AKT signaling [11,12].

In addition to PJS-related malignancies, *LKB1* is mutated in multiple sporadic cancers. Somatic mutations in *LKB1* occur in 5–17% of non-small cell lung carcinomas [5,13–16] and up to 5% of pancreatic cancers and melanomas [17–19]. A recent study by Wingo *et al* [20] of 86 primary cervical tumors demonstrated that at least 20% possessed somatically acquired *LKB1* mutations. Additionally, an independent study identified *LKB1* mutations in 6 of 19 (32%) patients with sporadic minimal deviation adenocarcinoma (MDA), a rare form of cervical cancer often observed in PJS patients [13,21]. Thus, accumulating evidence suggests a role for *LKB1* mutations in the etiology of cervical cancer.

Herein, we further investigate the status of the *LKB1* gene in cervical cancer. We find that half of the cervical cancer cell lines examined exhibit homozygous deletions affecting all or part of the *LKB1* gene. The HeLa cell line exhibited a homozygous deletion resulting from an Alu-recombination mediated deletion (ARMD) that generates a novel *LKB1* fusion transcript driven by an uncharacterized CpG island promoter located ~1 kb upstream of *LKB1*. Similarly, a homozygous deletion of ~75 kb in SiHa cells that removes the entire *LKB1* genes generates a fusion transcript derived from *SBNO2* and *c19orf26*, the two genes flanking *LKB1*. Finally, we utilize public databases of gene expression and mutation information to further estimate the extent to which this region is dysregulated in primary cervical cancers. We find that primary cervical cancers exhibit a high incidence of *LKB1* mutation and coordinated transcriptional down-regulation of *LKB1* and its neighboring genes *SBNO2* and *c19orf26*.

METHODS

Cell lines and cell culture

HeLa and HeLa S3 cervical adenocarcinoma cell lines were obtained from American Tissue Culture Collection (ATCC) and maintained in EMEM media containing 10% fetal bovine serum (FBS). C33A (carcinoma), CaSki (epidermoid carcinoma metastatic to small intestine), and SiHa (grade II squamous cell carcinoma) cervical cancer cell lines were kindly provided by Dr. Michelle Ozbun (University of New Mexico) and were maintained in either EMEM media (C33A, SiHa) or DMEM media (CaSki) containing 10% FBS.

Protein isolation and western blotting

Cells were lysed on ice for 1 hour in 3 cell pellet volumes of 50 mmol/L Tris-Cl (pH 8.0), 150 mmol/L NaCl, 0.5 mmol/L EDTA, 1% NP40, 0.5% sodium-deoxycholate, 0.1% SDS containing protease inhibitors (Roche). Cell debris was pelleted by centrifugation and the supernatant was isolated and quantified. Protein lysates (75 µg) were electrophoresed on a 12% SDS-PAGE gel and transferred onto a PVDF membrane (BioRad) for 1 hour at 120 volts. The membrane was then blocked for 1 hour at room temperature with 10% milk-TBST followed by overnight incubation with primary antibody in 5% milk-TBST. Membranes were washed in TBST, incubated for 1 hour with horseradish peroxidase-conjugated secondary antibody, washed again, and subjected to chemiluminescence detection (Pierce). Antibodies used were LKB1 (27D10, Cell Signaling) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Abcam).

RNA extraction and reverse transcription-PCR

Total cellular RNA was extracted from 1×10^7 cells with the RNeasy Mini kit (Qiagen, Valencia, CA) according to the manufacturer's directions. The resulting total RNA (3 µg) was treated with DNaseI and reverse transcribed using 300ng random hexamers in the presence of 1× First Strand buffer, 10mM DTT, 0.5mM dNTPs, 12U RNase-OUT, and 300U MMLV reverse transcriptase for 1 hour at 37°C. PCR was performed using 0.5U Taq DNA polymerase (Invitrogen), 0.4 µM gene-specific sense primer, 0.4µM gene-specific antisense primer, 0.5mM dNTPs, and 1 µL cDNA in 1× PCR buffer contain 3.0mM MgCl₂. Amplification proceeded as follows: initial denaturation at 94°C (3 minutes), 35 cycles of 94°C (30 seconds), 59°C (30–60 seconds), and 72°C (30–90 seconds), followed by a final extension at 72°C (5 minutes). PCR products were resolved on 1–2% agarose gels containing 0.5 µg/mL ethidium bromide in Tris-acetate EDTA (TAE) buffer. RT-PCR primer sequences are provided in Supplementary Table 1.

Methylation analysis

Genomic DNA was extracted from cell lines using the DNeasy Tissue Kit (Qiagen) and 1µg DNA was bisulfite-modified with the EZ DNA Methylation-Gold kit (Zymo Research) according to manufacturer's recommendations. Methylation-specific PCR (MSP) was performed with approximately 20ng of bisulfite-modified DNA as previously described [22, 23]. LKB1 MSP primers were: unmethylated sense 5'-TGGGTGGTTTGGAGAAGATTGT-3', unmethylated antisense 5'-CCCAACTACAAATCCAAACCCA-3', methylated sense 5'-GGCGGTTCCGGAGAAGATTGC-3', and methylated antisense 5'-CCGACTACGAATCCAAACCCG-3'. As a methylation positive control, genomic DNA was *in vitro* methylated with the bacterial DNA methyltransferase M.SssI (New England Biolabs) according to manufacturer's recommendations.

Deletion mapping via PCR

Genomic DNA was extracted from cell lines using the DNeasy Tissue Kit (Qiagen). PCR was performed using 0.5U Taq DNA polymerase (Invitrogen), 1µM gene-specific sense primer, 1µM gene-specific antisense primer, 0.1mM dNTPs, 10% DMSO, and 50ng genomic DNA in 1× PCR buffer containing 6.7mM MgCl₂. Amplification proceeded as follows: initial denaturation at 94°C (3 minutes), 35 cycles of 94°C (30 seconds), 59°C (45 seconds), and 72°C (1 minute), followed by a final extension at 72°C (5 minutes). PCR products were resolved on 1.2–2.0% agarose gels containing 0.5 µg/mL ethidium bromide in TAE buffer. Deletion mapping PCR primer sequences are provided in Supplementary Table 2.

Transcription start site analysis

The 5' RACE system for rapid amplification of cDNA ends (Invitrogen) was utilized to localize the transcription start site of the novel deletion-induced *LKB1* transcript in HeLa cells. An antisense gene-specific primer (2.5pmol; 5'-CAGCACACACCCGTCCTGGG-3') located immediately downstream of the deletion breakpoint was utilized along with Superscript II reverse transcriptase to generate gene-specific cDNA from 2µg total HeLa RNA according to the manufacturer's directions. Following cDNA purification with the SNAP column, a homopolymeric tail was added to the 3' end of the cDNA with terminal deoxynucleotidyl transferase (TdT). The 5' end of the deletion-induced transcript was then amplified using a gene-specific antisense primer (5'-AAGTCACAGAAGTCCAGGCAC-3') and the 5' RACE Abridged Anchor Primer (Invitrogen) which recognizes the dC-tailed cDNA. Amplification proceeded as follows: initial denaturation at 94°C (3 minutes), 35 cycles of 94°C (45 seconds), 61°C (45 seconds), and 72°C (2 minutes), followed by a final extension at 72°C (5 minutes). The resulting PCR products were cloned into the TOPO-TA vector (Invitrogen) and transformed into OneShot TOP10 bacteria according to the manufacturer's directions. Individual colonies (n=3) were cultured and plasmid DNA was purified with the Qiaprep Spin Miniprep Kit (Qiagen) prior to sequencing at the University of Michigan DNA Sequencing Core.

Computational analyses

Custom PERL scripts were utilized to calculate the moving average of Alu density surrounding the *STK11/LKB1* locus. Genome-wide Alu repeat location data was obtained from the University of California Santa Cruz (UCSC) Genome Browser RepeatMasker track for the NCBI36.1 (HG18) build of the human genome. Alignment of DNA sequences to the human genome was performed utilizing the UCSC BLAT program (<http://genome.ucsc.edu/>) and Sequencher (Gene Codes Corporation). RNA polymerase II occupancy was assessed in multiple cancer cell lines using the UCSC genome browser (<http://genome.ucsc.edu/>) to display ChIP-Seq data generated by Michael Snyder's group (Yale University) for the ENCODE project. *LKB1* mutation data from primary tumors was obtained from the Sanger Institute's Catalogue of Somatic Mutations in Cancer (COSMIC) database (<http://www.sanger.ac.uk/genetics/CGP/cosmic/>; version 41) [24].

RESULTS

Loss of *LKB1* expression in cervical cancer cell lines

The *LKB1* protein is encoded by a 22.6kb gene located on chromosome 19p13.3 and regulates cell growth and motility in normal cells. Inactivation of *LKB1* is a frequent event in cancer cells occurring through loss-of-function mutations, deletion, and occasionally DNA methylation-mediated silencing [13,14,25]. To investigate the status of *LKB1* in cervical cancer cell lines, we analyzed the expression of the *LKB1* protein and RNA transcript in several cervical cancer cell lines. Western blot and reverse-transcription PCR (RT-PCR) analyses revealed complete loss of the full-length *LKB1* protein and transcript in HeLa, HeLa S3, and SiHa cells (Figure 1A/B). Since *LKB1* promoter methylation has been associated with transcriptional silencing in colorectal, testicular, papillary breast [26], and pancreatic [27] cancers, we assessed the methylation status of a 2.4kb CpG island located between -700bp and +1700bp relative to the *LKB1* transcription start site (defined by the start of RefSeq NM_000455.4) (Supp. Figure 1). Methylation-specific PCR (MSP) demonstrated that *LKB1* is unmethylated in the C33A and CaSki cell lines, however, HeLa, HeLa S3, and SiHa cells generated no PCR products despite successful amplification of both unmethylated and methylated control DNAs. Lack of detectable transcripts and the inability to amplify this region from genomic DNA in the MSP analysis indicated the possibility of genetic alterations (e.g. homozygous deletion) in these cell lines.

Homozygous deletion of *LKB1* in cervical cancer cell lines

To investigate the status of the *LKB1* CpG island, genomic PCR was performed for several regions within the promoter and first exon of *LKB1*. This analysis revealed biallelic loss of the *LKB1* promoter region in SiHa, HeLa, and HeLa S3 cells (Figure 1C). To determine the extent of homozygous deletion in these cells, additional regions extending across the *LKB1* gene including upstream and downstream regions were analyzed. Utilizing this approach, the deletion present in SiHa cells was found to encompass a ~75kb region extending from the first intron of the *SBNO2* isoform 1 gene (~45kb upstream of the *LKB1* transcription start site (TSS)) through the *c19orf26* gene (~8kb downstream of the *LKB1* transcription termination site). The *SBNO2-1* (66.7kb), *LKB1* (22.6kb), and *c19orf26* (8.0kb) genes are contiguous and span a region of ~100kb on chromosome 19p13.3. The deletions present in HeLa and HeLa S3 cells were similarly mapped to a ~24kb region beginning ~10.5 kb upstream of the *LKB1* TSS through intron 3 of *LKB1* and did not affect the coding region of any neighboring genes.

Generation of a *c19orf26-SBNO2* fusion transcript in SiHa cells

To precisely map the breakpoints of the deletion observed in SiHa cells, we amplified across the deletion with PCR (Figure 2A/B). This approach generated a specific PCR product in SiHa cells, but not in the other cell lines where the product would be expected to be ~76kb for the wild-type locus (Figure 2B). Sequencing of this product indicated that the first intron of *SBNO2-1* (chr19:1,111,208) was fused to the first intron of *c19orf26* (chr19:1,187,509). Thus, the homozygous deletion observed in SiHa cells removed the first exon and promoter region of the *SBNO2* isoform 1 gene, the entire *LKB1* locus, and all but the first exon and promoter region of *c19orf26*. The boundaries of this deletion differ from that recently report by Wingo *et al* [20], who suggested that SiHa cells harbor a deletion that only affects *SBNO2-1* and *LKB1*.

Since *SBNO2-1* and *c19orf26* genes are transcribed from the same strand of the chromosome and the first exons of both genes are non-coding, it is possible that a fusion transcript driven by the *c19orf26* promoter and encoding the entire wild-type *SBNO2-1* coding sequence could be generated. RT-PCR analysis revealed that both genes were expressed in cervical cancer cells with a wild-type *LKB1* locus and in HeLa cells in which the deletion did not extend into these genes. SiHa cells, however, expressed a spliced *c19orf26-SBNO2-1* fusion transcript driven by the *c19orf26* promoter (Figure 2C/D). In order to compare the relative abundance of this fusion transcript to that of wild-type *SBNO2*, we performed RT-PCR with primers located in *SBNO2* exons 2 and 5 which are present in both wild-type *SBNO2* and the *c19orf26-SBNO2* fusion transcripts (Figure 2C). Interestingly, the *c19orf26* promoter appears to maintain expression of the *SBNO2-1* coding sequence at levels approximating those observed in cervical cancer cell lines with an intact *SBNO2* gene. However, while basal transcription of the *c19orf26-SBNO2* fusion transcript in SiHa cells may be similar to that of wild-type *SBNO2*, the *c19orf26* promoter may respond differently than the *SBNO2* promoter to cellular perturbations.

Homozygous deletion of *LKB1* in HeLa cells via an aberrant Alu recombination event

The breakpoints of the smaller *LKB1* deletion occurring in HeLa and HeLa S3 cells were also mapped via PCR (Figure 3A). Sequencing of the PCR products revealed identical homozygous deletions which brought together an intergenic region located ~11kb upstream of *LKB1* with intron 3 of *LKB1*. The presence of identical deletion breakpoints in HeLa and HeLa S3 cells indicates that this alteration occurred prior to the sub-cloning of the HeLa S3 cell line from HeLa cells in 1955 [28] and suggests that it may derive from the primary tumor from which the HeLa cell line was established. Indeed, Wingo *et al.* recently demonstrated the presence of an *LKB1* homozygous deletion in the primary tumor from which the HeLa cell line was propagated [20].

Detailed examination of the sequence alignment of the HeLa trans-deletion PCR product to the genome revealed a 45bp length of perfect homology between the upstream and downstream breakpoints (Figure 3B). An extended alignment of sequences upstream and downstream of the breakpoints revealed ~280 bp that shared 87% identity. This region of identity corresponded to AluY repetitive elements (AluYb8 upstream; AluY downstream) suggesting that this deletion likely occurred through an Alu recombination-mediated deletion (ARMD). ARMDs result from mispairing of Alu elements located either intra-chromosomally or inter-chromosomally during meiosis and have contributed to genomic variation throughout evolution [29, 30]. ARMDs occur most frequently between younger Alu repeats (i.e. AluY) oriented in the same direction in Alu-dense regions [30, 31]. Consistent with these findings, the deletion observed in HeLa cells occurred in an Alu-dense region (Figure 3C) between two highly homologous AluY repeats (Figure 3D) both located in the (–) orientation relative to the chromosome. Thus, the unique composition of repetitive elements surrounding the *LKB1* locus likely contributed to the deletion of the *LKB1* promoter and first three exons in HeLa cells.

Generation of a novel *LKB1* fusion transcript in HeLa cells

The intergenic region upstream of the homozygous deletion observed in HeLa cells contains an uncharacterized 1.6kb CpG island (Figure 1C). Since CpG islands often co-occur with gene promoters, we utilized computational tools to assess the transcriptional potential of this region. The ESPERR (Evolutionary and Sequence Pattern Extraction through Reduced Representations) program aims to discriminate regulatory regions from neutral sites using DNA sequence patterns and measures of evolutionary conservation [32]. This program revealed a focal region of elevated regulatory potential for this CpG island (chr19:1145702-1145758; Supp. Figure 2). Similarly, using the FirstEF (First Exon Finder) 5' terminal exon and promoter prediction program [33], a portion of this CpG island (chr19:1145389-1145958) is predicted to have promoter and first exon potential (Supp. Figure 2). It is noteworthy, that in the 31.5kb of intergenic sequence located between *SBNO2* and *LKB1* this is the only region with predicted promoter activity. We then searched for predicted transcription start sites (TSS) using the Eponine program which was trained to identify TSSs using DNA patterns and their positions relative to a set of known TSSs [34]. Eponine predicted a TSS (chr19:1145686-1145694) within the same region predicted to have promoter and regulatory potential (Supp. Figure 2).

To examine the transcriptional status of this CpG island, we assessed RNA polymerase II (PolII) occupancy utilizing a dataset from the Encyclopedia Of DNA Elements (ENCODE) project. This dataset included genome-wide localization of PolII occupancy using chromatin immunoprecipitation-massively parallel sequencing (ChIP-Seq) technology in the immortalized HEK293 (embryonic kidney) cell line and four cancer cell lines, including HeLa, GM12878 (lymphoblastoid), K562 (chronic myelogenous leukemia), and NB4 (acute promyelocytic leukemia). Although no genes or expressed sequence tags (ESTs) have been reported to overlap this CpG island, a peak of PolII was observed over the region in 4 of the 5 cell lines including HeLa (Supp. Figure 3). This analysis revealed two additional findings. First, the homozygous deletion in HeLa cells can easily be observed in this dataset based on the lack of sequence tags mapping to this region. Second, HeLa cells exhibit elevated PolII binding at the upstream CpG island relative to the other cell types, as well as increased PolII binding over a larger region covering at least 100kb relative to cell lines with an intact *LKB1* locus.

To directly assess whether transcripts arise from this region in wild-type or deleted cells, we performed RT-PCR using primers from within this CpG island (chr19:1145467-1145749) (Figure 4A). RNA products were detected in C33A, CaSki, HeLa, and HeLa S3 cells. The absence of PCR products in controls lacking reverse-transcriptase (RT) and in cDNA from

SiHa cells confirms that this product is not the result of DNA contamination or non-specific amplification. Thus, this region upstream of *LKB1* is transcriptionally active in both cervical cancer cells with an intact *LKB1* locus and in HeLa cells.

Although the homozygous deletion present in HeLa cells removes the promoter and first three exons of *LKB1*, it is possible that bringing the transcriptionally competent region upstream of the deletion into proximity with *LKB1* exons 4–10 might generate a fusion transcript. Despite the absence of *LKB1* promoter and 5' exons, RT-PCR analysis of *LKB1* exons 4 through 10 revealed transcripts containing these regions in HeLa and HeLa S3 cells, albeit at a reduced level relative to C33A and CaSki cells (Figure 4B). To determine whether this partial *LKB1* transcript was derived from the upstream CpG island, RT-PCR was performed with one primer located within the upstream CpG island and the other in exon 4 of *LKB1*. A transcript spanning the deletion was observed only in the HeLa cell lines, and not in the C33A or CaSki cell lines in which *LKB1* is intact (Figure 4B). In addition, cloning and sequencing of a longer RT-PCR product with primers located within the CpG island and *LKB1* exon 6 revealed a transcript that included the upstream CpG island, the remaining portion of intron 3, and exons 4 through 6 with splicing out of introns 4 and 5 (Figure 4C). In order to determine the TSS of this transcript, we used 5' rapid amplification of cDNA ends (5'-RACE) with a gene-specific primer located immediately downstream of the deletion breakpoint. This approach identified two unique transcription start sites within a 7bp window located in the upstream CpG island (Figure 4C/D; Supp. Figure 4). This window of transcription start sites was located 8bp downstream of the Eponine predicted TSS. Thus, the homozygous deletion involving the *LKB1* locus in HeLa cells generates a novel fusion transcript derived from an upstream CpG island promoter (Figure 4E).

Based on the RT-PCR and 5'-RACE results, the anticipated full-length fusion transcript would be ~2.6kb comprised of ~350bp upstream sequence, ~650bp from *LKB1* intron 3, and ~1.7kb from *LKB1* exons 4–10 (Figure 4E; Supp. Figure 4). This transcript has a predicted open reading frame (ORF) that starts with an ATG within *LKB1* intron 3 and continues in-frame with *LKB1*. This transcript thus has the potential to encode a 358 amino acid (AA) protein composed of 79 AA of novel sequence from intron 3 with no homology to known protein domains fused to the C-terminal 279 AA of *LKB1* (Figure 4E; Supp. Figure 5). This deletion removes the first ~100 AA of the *LKB1* kinase domain and likely abrogates the protein's kinase activity. However, it remains unclear whether this novel fusion transcript forms a stable protein in HeLa cells as we have been unable to detect a protein of the anticipated molecular weight using several commercially available antibodies (data not shown).

***LKB1* transcriptional repression in cervical cancers**

These analyses have revealed large homozygous deletions in half of the cervical cancer cell lines tested. To approximate the rate of *LKB1* mutation in primary cervical cancers, we mined the Sanger Institute's Catalogue of Somatic Mutations in Cancer (COSMIC) database for alterations within the *LKB1* gene in cervical cancer. Within this dataset, *LKB1* was one of the most frequently mutated genes reported for cervical cancer with 13 of 108 samples tested (11%) exhibiting coding region mutations. Additionally, among the 32 cancer types for which data exist, cervical cancer had the 3rd highest frequency of *LKB1* mutation along with lung cancer (109 of 1002; 11%) and was surpassed only by tumors of the gastrointestinal tract (5 of 21; 24%) and prostate (1 of 5; 20%). The mutations reported in cervical cancers consisted exclusively of point mutations leading to amino acid substitutions. Thus, *LKB1* is frequently targeted for mutation in primary cervical tumors further supporting a role for this tumor suppressor gene in cervical cancers.

In addition to loss-of-function mutations and genomic deletions, transcriptional repression can also contribute to the inactivation of tumor suppressor genes. Analysis of the Oncomine

database [35] of cancer transcriptome profiles revealed that *LKB1* exhibits decreased expression in cervical cancers (Figure 5A) [36], adult male germ cell tumors [37], and advanced stage ovarian carcinomas relative to their normal counterparts. In the case of cervical cancers, 16 of 20 tumors exhibited lower *LKB1* expression than the lowest expression observed in normal samples. The same study also showed decreased expression of *SBNO2* and *c19orf26*, the two genes flanking *LKB1*, in cervical cancers when compared to normal cervix samples (Figure 5B/C). Cases with coordinated down-regulation of *SBNO2*, *LKB1*, and *c19orf26* might suggest larger alterations at the *LKB1* locus, similar to the homozygous deletion shown here for SiHa cells. In 8 of 20 (40%) cervical cancers, all 3 genes were down-regulated and in 14 of 16 (87.5%) cases where *LKB1* was down-regulated either *SBNO2* or *c19orf26* was also down-regulated (Figure 5D). The independent findings that *LKB1* is affected by both frequent mutation at the DNA level and by transcriptional down-regulation suggest that inactivation of *LKB1* is an important contributing factor in the pathogenesis of cervical cancers.

DISCUSSION

The *LKB1* kinase is an essential regulator of cell growth, proliferation, and polarity in normal human cells. Disruption of the *LKB1* gene is clearly linked to the development and progression of several types of human cancers as observed in both PJS and sporadic tumors. Utilizing genomic profiling of the *LKB1* locus and surrounding regions, we have identified two homozygous deletions of 25kb and 85kb which generate unique fusion transcripts in commonly studied cervical cancer cell lines. Identification of these deletions in cervical cancer cells further supports a role for *LKB1* disruption in the development of this disease and highlights the complex effects that it has on transcriptional regulation of the affected genes.

The majority of reported *LKB1* mutations are point mutations or single base deletions/insertions which cause loss of function or frameshifts leading to premature truncation [25,38]. The highest rate of *LKB1* mutations within the cervix has been reported for sporadic cases of MDA (32%; 6 of 19), a rare sub-type of cervical cancer for which PJS patients are at increased risk [21, 39]. To date, seven point mutations have been reported in cervical adenocarcinomas (n=5) and squamous cell carcinomas (n=2) with many of these affecting phylogenetically conserved residues within the kinase domain or intron/exon boundaries [13,20,21]. Larger homozygous deletions of *LKB1* have less frequently been reported with a few identified in pancreatic [17], biliary [17], and lung [16] cancers. Wingo *et al* recently reported five large homozygous deletions in cervical adenocarcinomas (n=1) and squamous cell carcinomas (n=4) along with 4 single copy losses within this region (n=1 adenocarcinoma, n=3 squamous cell carcinoma) [20]. Thus, large homozygous deletions are an additional mechanism for loss of *LKB1* in the most common forms of cervical cancer.

Interestingly, while we identified two unique homozygous deletions of the *LKB1* gene, we also identified two cervical cancer cell lines (C33A and CaSKI) that did not exhibit large-scale alterations of the *LKB1* coding sequence. These cell lines may either have inactivated another component of the *LKB1* pathway or they may have evolved along a route that affects another intersecting pathway resulting in similar downstream consequences. For example, there is evidence to suggest that alterations that promote PI3-Kinase/Akt pathway activation (*e.g.* activating mutations in *PIK3CA*, inactivation of *PTEN*) exhibit a mutually exclusive relationship with *LKB1* alterations [12]. This is consistent with the idea that the *LKB1* pathway and PI3 kinase/AKT play opposing roles in the downstream activation of mTOR signaling [12]. Consistent with this hypothesis, both C33A and CaSKi cell lines have been reported to harbor known gain-of-function mutations in *PIK3CA* [40], whereas none of the other five cervical cancer cell lines identified to have *LKB1* deletions (this report and [20]) were altered at *PIK3CA*. Moreover, of the 14 cervical cancer cell lines for which *LKB1* and *PIK3CA* mutation data are available from the Cancer Genome Project (COSMIC, [24]), 10 show

alterations in either *LKB1* (n=6) or *PIK3CA* (n=4) and the remaining four are wild-type for both. These data suggest that most cervical carcinomas exhibit dysregulation of mTOR signaling, but achieve this in different ways.

The molecular mechanisms underlying the generation of large deletions, such as those observed at the *LKB1* locus in HeLa and SiHa, are poorly understood. Non-random chromosomal rearrangements, such as those that occur in hematologic cancers, have been attributed to aberrant V(D)J recombination, Alu-mediated homologous recombination, cleavage at Z-DNA, fragile sites, repair of DNA breaks by nonhomologous end joining, and other mechanisms [41]. Since several of these mechanisms employ recombination, the sheer number of highly conserved *Alu* repetitive elements within the genome ($n > 10^6$) makes them a likely place for aberrant homologous recombination events [42]. The consensus *Alu* repeat is a 282bp bipartite element with a central A-rich region and flanking direct repeats derived from the site of insertion [29]. A bioinformatic search for Alu recombination-mediated deletions (ARMDs) occurring during evolution identified nearly 500 human-specific events in which *Alu* recombinations induced genomic deletions totaling ~400kb of sequence [30]. In addition to driving genome evolution, ARMDs also contribute to human disease. ARMDs occurring within the germline have been observed in several inherited disorders including α -thalassaemia, familial hypercholesterolaemia, Lesch–Nyhan syndrome, and Tay–Sachs disease [42], and have been implicated in the inactivation of tumor suppressor genes in familial cancers including *CDH1* in hereditary diffuse gastric cancer [43], *MLH1* in hereditary nonpolyposis colorectal cancer [44], *MEN1* in multiple endocrine neoplasia type 1 [45], and *BRCA1* in hereditary breast and ovarian cancer [46]. Alternatively, ARMDs of tumor suppressor genes have also been observed to occur somatically in tumor tissues such as *TMPRSS2/ERG* in prostate cancer [47], *CAD* in hepatoma [48], and *TSC2* in tuberous sclerosis [49].

Based on computational studies of ARMD events occurring during evolution, ARMDs preferentially occur between the younger and more conserved *AluY* elements in regions of high *Alu* density [30,31]. In the case of *LKB1* in HeLa cells, the ARMD occurred between two *AluY* elements with 87% overall identity, including a 45bp stretch of 100% identity, and the region between *SBNO2* and *LKB1* exhibits a density of *Alu* elements that is more than double the chromosomal average. Lastly, ARMDs occur more often between *Alu* elements arranged in the same orientation along the chromosome as is the case for the two *AluY* elements involved in the HeLa ARMD [30]. With many other highly-related *Alu* repeats located within this chromosomal location, it is likely that other combinations of *Alu* elements may recombine to affect the *LKB1* locus in human cancers.

Genomic rearrangements generating novel fusion transcripts were originally reported in hematological malignancies, but have now been described in several common solid tumor types [50–52]. While the true prevalence of fusion transcripts in cancer cells is still being elucidated, recent approaches utilizing massively parallel sequencing of cancer transcriptomes have computationally identified and experimentally validated several novel chimeric transcripts in solid tumors [53,54]. In this study, we have identified two novel fusion transcripts arising as a consequence of *LKB1* deletion. In SiHa cells, an intrachromosomal deletion generated a fusion transcript containing the first exon of *c19orf26* and all but the first exon of *SBNO2*. As the coding sequence of both of these genes begins in their second exon, this rearrangement did not affect the coding sequence of *SBNO2*, but did replace the *SBNO2* regulatory sequences with those of *c19orf26*. Based on RT-PCR analyses, the *c19orf26*-*SBNO2* fusion does not appear to be expressed at a considerably different level than the wild-type *SBNO2* transcript. However, the *c19orf26* promoter is likely regulated differently than the *SBNO2* promoter which may have important consequences for cancer progression.

The deletion observed in HeLa cells also generates a novel fusion transcript, but with a previously uncharacterized region of transcriptional potential ~11kb upstream of the *LKB1* TSS. Similar to the *c19orf26-SBNO2* fusion observed in SiHa cells, a portion of the *LKB1* gene is placed under the control of a novel regulatory element which may have important consequences for expression of this fusion transcript. While the homozygous deletion reported in SiHa cells affects the entire *LKB1* gene, the deletion observed in HeLa cells only affects the first three exons. Together these exons encode the 155 N-terminal AA of LKB1 comprising approximately the first 100 AA of the kinase domain including the ATP-binding domain, but not the kinase active site which is located between residues 172 and 184. The deleted region of *LKB1* also encodes a nuclear localization signal at AA38-43 [38] and a portion of the domain required for interaction with Brg1 and repression of cell cycle [55]. Due to an ATG located within the 3rd intron of *LKB1*, the fusion transcript observed in HeLa cells has a predicted ORF including 79 AA of novel sequence fused to the C-terminal 279 AA of LKB1. As noted above, if this transcript generates a stable protein product it is expected to lack kinase activity. However, such a product could interact with other LKB1-interacting partners and potentially interfere with LKB1 function through dominant-negative effects. Additional work will be necessary to determine if this fusion transcript generates a stable protein product and its impact on the integrity of the LKB1 signaling pathway. Since the HeLa cell line is frequently utilized to study the effects of exogenous LKB1, it will be important to examine the potential impact of this partial transcript on full-length LKB1.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Full-length *LKB1* is not expressed in some cervical cancer cell lines due to homozygous deletion

A, Western blot and B, RT-PCR analyses demonstrate lack of LKB1 protein and full-length mRNA transcript in HeLa, HeLa S3, and SiHa cells. GAPDH and actin are included as loading controls and a reverse transcriptase negative (RT(-)) panel is included for actin to demonstrate complete removal of genomic DNA. C, Genomic PCR (gPCR) performed at multiple loci across the *SBNO2-LKB1-c19orf26* region demonstrates homozygous deletion of the first three exons of *LKB1* in HeLa and HeLa S3 cells and the first exon of *SBNO2*, all of *LKB1*, and all but the first exon of *c19orf26* in SiHa cells. The location of CpG islands (CpGi; green) and gPCR products (gray) are indicated above and below the gene tracks (blue), respectively.

Figure 2. Characterization of a *c19orf26-SBNO2* fusion transcript in SiHa cells

A, Schematic of PCR primer sets used for breakpoint cloning and RT-PCR. CpG islands and RefSeq genes are shown in green and blue, respectively. B, Genomic PCR demonstrating continuity across the deleted region in SiHa cells. PCR products were purified, cloned, and sequenced to precisely define the deletion boundaries. C, RT-PCR analysis of *SBNO2*, *c19orf26*, and the *c19orf26-SBNO2* fusion transcripts in cervical cancer cell lines. A set of primers from *SBNO2* exons 2 and 5 were utilized to compare *SBNO2* expression between intact and deleted cells. Actin is included as a control. D, Schematic of the *c19orf26-SBNO2* fusion transcript with exon numbers indicated within each box and translation start (ATG) and termination (TGA) codons shown.

Figure 3. Deletion of *LKB1* in HeLa cells via an illegitimate Alu-recombination

A, Genomic PCR demonstrating continuity across the deleted region in HeLa cells. PCR products were purified, cloned, and sequenced to precisely define the deletion boundaries. B, Alignment of the sequences immediately upstream and downstream of the deletion with the sequenced trans-deletion PCR product from HeLa S3. Bases that are not conserved in either the upstream or downstream regions relative to the PCR product are in bold. A 45bp region of perfect identity is enclosed in a dashed box. C, Relative density of Alu repeats in the region overlapping and surrounding the *LKB1* deletion observed in HeLa cells (box with diagonal lines). The average Alu density for chromosome 19 is depicted by the dashed horizontal line. Individual SINE elements (black), CpG islands (green), and RefSeq genes (blue) are indicated below the graph. D, Homology alignment scores for all Alu repeats within the region spanning 15kb upstream of *LKB1* to the transcription termination site of *LKB1*. The Alu located within the 3rd intron of *LKB1* that is involved in the deletion serves as the reference sequence. Alignment scores are plotted for each Alu shown and are plotted in the same order that they occur along the chromosome. Lines connect the Alus involved in the *LKB1* deletion to their respective data bars that are highlighted in black.

Figure 4. HeLa cells express an *LKB1* fusion transcript derived from an upstream CpG island RT-PCR analysis of A, the upstream CpG island, B, 3' exons of *LKB1*, *LKB1* fusion transcript, and actin in cervical cancer cell lines. C, Diagram depicting the sequence alignment of cloned RT-PCR and 5' RACE products to the *LKB1* gene (blue) and CpG islands (green). D, Genomic alignment of the 5' ends of the unique 5' RACE products. E, Schematic representing the predicted *LKB1* fusion transcript expressed in HeLa cells with exon numbers indicated within each box and potential translation start (ATG) and termination (TGA) codons shown.

Figure 5. The *SBNO2-LKB1-c19orf26* locus is transcriptionally repressed in primary cervical cancers

Gene expression levels were assessed for A, *LKB1*, B, *SBNO2*, and C, *c19orf26* in a published dataset of 8 normal cervix (green) and 20 cervical cancer samples (red) [36] using the Oncomine database. The top and bottom of the boxes represent 25th and 75th percentile values, the horizontal bar within the box represents the median value, and the whiskers represent the minimum and maximum values. D, Venn diagrams representing the overlap between tumors exhibiting down-regulation of *LKB1* (red) and either *SBNO2* (green) or *c19orf26* (blue). Genes were defined as down-regulated if their expression value was less than the lowest value recorded among the normal samples.