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## Frequent homozygous deletion of the *LKB1/STK11* gene in non-small cell lung cancer

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### Abstract

*LKB1/STK11* is a tumor suppressor and a negative regulator of mammalian target of rapamycin signaling. It is inactivated in 30% of lung cancer cell lines but only 5–15% of primary lung adenocarcinomas. There is evidence that homozygous deletion (HD) of chromosome 19p at the *LKB* locus contributes to the inactivation of the gene in primary human lung cancers. Here, we used several complementary genetic approaches to assess the *LKB1* locus in primary non-small cell lung cancers (NSCLCs). We first analyzed 124 NSCLC cases for allelic imbalance using eight microsatellite markers on chromosome 19p, which revealed an overall rate of 65% (80 of 124) loss of heterozygosity (LOH). We next used chromogenic *in situ* hybridization (CISH) to directly examine the chromosomal status of the *LKB1* locus. In all, 65 of 124 LOH tested samples were available for CISH and 58 of those (89%) showed either loss of one copy of chromosome 19p (LOH, 40 of 65 cases, 62%) or both copies (HD 18 of 65 cases, 28%). The occurrence of HD was significantly more frequent in Caucasian (35%) than in African-American patients (6%) ( $P=0.04$ ). A total of 62 of 124 samples with LOH at one or both markers immediately flanking the *LKB1* gene were further analyzed by directly sequencing the complete coding region, which identified 7 of 62 (11%) tumors with somatic mutations in the gene. Jointly, our data identified total inactivation of the *LKB1* gene by either HD or LOH with somatic mutation in 39% of tested samples, whereas loss of chromosome 19p region by HD or LOH at the *LKB1* region occurred in 90% of NSCLC.

### Keywords

*LKB1* gene; LOH; homozygous deletion; mutation; CISH; primary lung cancer

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### Conflict of interest

The authors declare no conflict of interest.

## Introduction

The *LKB1* gene is a tumor suppressor gene located on chromosome 19p13.3 (Hemminki *et al.*, 1998). It is also known as *STK11*, has serine–threonine kinase activity and possesses nuclear localization signals in the N-terminal region, central kinase domain and a C-terminal farnesylation region (Alessi *et al.*, 2006). The *LKB1* gene is widely expressed at varying levels in all fetal and adult tissues. During development, expression of LKB1 is high in the heart, esophagus, pancreas, kidney, colon, lung, small intestine and stomach (Luukko *et al.*, 1999; Rowan *et al.*, 1999). In adult tissues, expression is high in most epithelium of follicles, including the corpus luteum of the ovary and the seminiferous tubules of the testis, in myocytes from skeletal muscle, and in glia cells (Rowan *et al.*, 1999; Conde *et al.*, 2007).

Functionally, LKB1 regulates cellular energy metabolism and cell polarity by activating AMP-activated protein kinase (AMPK) and other members of the AMPK family (Hawley *et al.*, 2003; Woods *et al.*, 2003; Corradetti *et al.*, 2004; Shaw *et al.*, 2004a, b). Activation of AMPK restores ATP levels by inducing catabolic pathway-like glycolysis and blocking ATP-consuming anabolic pathways—like protein synthesis—thus acting as a sensor of intracellular ATP levels. AMPK controls protein synthesis by regulating the mammalian target of rapamycin through phosphorylation of tuberous sclerosis protein 2 (TSC2), also known as tuberin (Inoki *et al.*, 2003). Deletion of LKB1 results in the loss of AMPK functions and improves glucose uptake (Koh *et al.*, 2006). In the liver, LKB1 knockout causes hyperglycemia due to increased expression of the gluconeogenic gene and hepatic glucose output (Shaw *et al.*, 2005). More recently, genetic and functional studies have demonstrated a critical role of LKB1 in regulating cell polarity and provided an underlying mechanism in colonic polyp development in patients with Peutz–Jeghers syndrome (Zhang *et al.*, 2008; Jansen *et al.*, 2009).

Genetically, mutations in the *LKB1* gene are responsible for Peutz–Jeghers syndrome, an autosomal dominant disorder characterized by melanocytic macules of the lips, buccal mucosa, and digits, and multiple gastro-intestinal hamartomatous polyps. Patients with Peutz–Jeghers syndrome have a high risk of gastrointestinal and other cancers (Lim *et al.*, 2003). In addition, cumulative evidence suggests that somatic mutation of *LKB1* gene is critically involved in lung and cervical cancers (Sanchez-Cespedes *et al.*, 2002; Wingo *et al.*, 2009). The involvement of LKB1 in lung cancer is several fold. First, chromosome 19p is the second most common region of loss in lung tumors of smokers (Sanchez-Cespedes *et al.*, 2001). Second, inactivation mutations of LKB1 are found in 30% of lung cancer cell lines and in a smaller portion of primary lung adenocarcinomas (Sanchez-Cespedes *et al.*, 2002; Davies *et al.*, 2005). Most somatic mutations involve nonsense or frameshift mutations leading to a truncated protein (Launonen, 2005; Shaw *et al.*, 2005). Third, transgenic animal studies suggest that LKB1 modulates lung cancer differentiation and metastasis (Gurumurthy *et al.*, 2008). Finally, LKB1 regulates lung cancer cell polarity, and deficiency in LKB1 sensitizes mice to carcinogen-induced tumorigenesis (Zhang *et al.*, 2008). In cervical cancer, point mutation and/or deletion of the *LKB1* gene was observed in 20% of the primary cervical tumor samples. Deletions were found in about 50% of the cervical cancer cell lines, such as HeLa and SiHa. Furthermore, chromosomal loss or mutation of the *LKB1* gene is associated with a reduced progression-free survival in patients (Wingo *et al.*, 2009). Taken together, these data further support a critical role of LKB1 in tumorigenesis and progression.

To gain insights into the mechanism of *LKB1/STK11* gene alterations in non-small cell lung cancer (NSCLC), we utilized several complementing genetic approaches, including allelic analysis for chromosomal imbalance or loss (loss of heterozygosity (LOH)), sequencing for tumors with LOH, and chromogenic *in situ* hybridization (CISH) to investigate the genetic

status of the gene at a single cell level. Consistent with others, we observed a generally low rate of somatic mutations (11%) at the *LKB1* gene. However, we observed that LOH and homozygous deletion (HD) of the *LKB1* gene jointly occurred in nearly 90% of the NSCLC tested.

## Results

### Status of chromosome 19p in NSCLC

We first examined the status of chromosome 19p in 124 lung cancer samples using a panel of eight microsatellite markers (Figure 1a). When considered as a whole, 65% (80 of 124) of the tumors showed allelic imbalance or LOH in two or more markers. When the presence of LOH at each marker was considered, the rate of loss at the marker closest to *LKB1*/*STK11*, D19S883, was the lowest (38%, Figure 1b), suggesting a possible mechanism for *LKB1* gene inactivation through HD in the region. Tumors from patients with stage I disease (71%) had a higher rate of LOH compared with those with stage II–IV disease (53%) ( $P=0.04$ , Table 1).

### *In situ* hybridization analysis of chromosomal status in NSCLC

To directly examine the status of chromosome 19p, we identified a bacterial artificial chromosome (BAC) clone, CDT-2315C07, located near the 3' end of the *LKB1* gene that was uniquely hybridized to the target region (Figure 1a). We developed a CISH method to directly examine the hybridization signals of the BAC clone in tissue samples using the lung tissue microarray (TMA) (examples in Figure 2). In total, 65 of 124 cases analyzed by microsatellite markers were available for analysis by CISH. The distribution of hybridization signals from CISH in cells from the non-tumor lung, adjacent non-involved lung, and lung tumor are summarized in Table 2. Non-tumor lung and adjacent normal lung tissues had a major portion (mode) of signals that were typical of a non-neoplastic tissue, primarily having two signals per cell in 76–86% of nuclei, whereas 3–10% had three to four signals per cell. Among the 65 lung tumors analyzed by CISH, 40 cases (61%) had only one signal per cell, consistent with LOH, whereas 18 cases (28%) had no hybridization signals in most tumor cells, suggesting HD. The remaining seven cases (11%) had approximately 50% of the cells having two signals per nuclei and were considered as no LOH.

We compared the results from CISH with those obtained by microsatellite analysis (Table 3). Of the 56 cases that were informative for D19S883, 21 of 22 cases having LOH had a loss of one signal in a majority of the cells ( $P<0.0001$ ). In contrast, of the 34 tumors that were considered as 'normal' at marker D19S883, 15 (44%) tumors had loss of both alleles by CISH, consistent with having HD, and 13 additional tumors (38%) had a loss in one of the *LKB* alleles. On the basis of CISH, only 7 of 34 (20%) remaining tumor samples showed no loss and were considered as 'normal,' and, of those, 6 also showed no loss by microsatellite marker D19S833. The comparison of LOH data obtained by marker D19S814 with CISH showed a similar trend in that all 26 tumors scored as LOH by microsatellite analysis were confirmed by CISH as either LOH or HD, whereas most of the 25 samples that were 'normal' by microsatellite analysis were either LOH (11) or HD (9) by CISH ( $P=0.003$ ). These results suggest that the CISH method is much more sensitive in detecting chromosome 19p losses when compared with those observed by the standard LOH method. Furthermore, no signal amplification was observed in any of the analyzed tumors further supporting a critical loss of genetic material in this region in NSCLC, and only 10% of the tumors had no loss at the *LKB* gene locus by both LOH and CISH analysis.

### Somatic mutations in the *LKB1* gene

Of the 124 tumors analyzed, 62 samples had LOH at either one or both microsatellite markers (D19S814, D19S883) flanking the *LKB1*/*STK11*. We sequenced all eight coding exons in these 62 tumors and identified seven tumors (11.3% overall) with a mutation in the *LKB1* gene (Table 4). All mutations were detected in the tumors from female patients who were either current or former smokers. Four cases were from African Americans and three were from Caucasians. Histologically, six tumors were adenocarcinomas and one was a squamous carcinoma. The clinical characteristics of all patients and their molecular changes in the mutated *LKB1* gene are summarized in Table 4. In total, we identified five cases with point mutations, three predicted to result in amino acid substitutions, two nonsense mutations, and two with single base deletions resulting in a frameshift, leading to premature termination and truncation of the protein. No mutations were found in the paired normal tissues, indicating that these mutations were somatic.

### Association of *LKB1* status in tumors with clinical characteristics of the patient

Overall, CISH analysis identified 89% (58 of 65) of the tested tumors with loss of at least one copy of chromosome 19p. Of those, 18 of 65 (28%) tumors lost both alleles, whereas 40 of 65 (62%) tumors lost one copy of the chromosome. Among the samples included in this study, there was a significant racial difference in loss of *LKB1*/*STK11* with 35% of the Caucasians and 6% of the African Americans having HD, 56% of the Caucasians and 77% of the African Americans having LOH, and 8% of the Caucasians and 17% of the African Americans having no LOH ( $P=0.04$ ), as shown in Table 1. Overall, 39% of primary NSCLC tumors analyzed had a complete inactivation of the gene through either loss of both copies of the *LKB1*/*STK11* gene or through LOH in one allele and somatic mutation in the remaining allele of the gene. Although not reaching statistical significance, patients with HD and mutations in lung tumors had worsened lung cancer prognosis compared with those whose tumors had only LOH ( $P=0.06$ , and data not shown). The survival comparison could not be done between patients whose tumor had mutation and those who did not have LOH due to the small sample numbers in each group.

### Discussion

In the present study we used three complementing genetic methods to assess genetic alteration in 19p13.3, and specifically the *LKB1*/*STK11* locus. We documented conclusively the high frequency of loss in this region in NSCLC. Our results support previous work, suggesting a high incidence of cytogenetic abnormalities at 19p in NSCLC (Lukeis *et al.*, 1990) and SCLC (Levin *et al.*, 1995). Sanchez-Cespedes *et al.* (2002) later showed that a chromosome 19p region, mapped between the telomere and microsatellite marker D19S216, was frequently associated with lung adenocarcinoma with an overall rate of 70% LOH (21 of 30 cases). The frequency of LOH (62%) in our data is consistent with the rate of 58% (15 of 26 cases) LOH observed by Sanchez-Cespedes *et al.* (2001). Subsequent studies led to the discovery that *LKB1* is a genetic target for lung cancer, particularly lung adenocarcinoma (Virmani *et al.*, 1998; Sanchez-Cespedes *et al.*, 2001; Carretero *et al.*, 2004; Matsumoto *et al.*, 2007). However, the rate of inactivating mutations in the gene remains relatively low and a high rate of LOH does not equate to inactivation of the candidate gene in the region. Another candidate tumor suppressor gene, *BRG1*, located about 10Mb proximal to the gene, can potentiate lung tumor development and is mutated in approximately 20% of the lung cancer cell lines (Glaros *et al.*, 2008; Medina *et al.*, 2008). The high rate of LOH that we observed here and by others strongly suggests that *BRG1* could also be a target of inactivation in this region.

We developed the CISH approach to investigate the genetic status of the region immediately adjacent to the *LKB1* gene by directly visualizing the chromosomal status in individual tumor cells. Among the 65 tumor samples that were analyzed by both methods, a majority showed loss of either one copy (62%, 40/65) or both copies (28%, 18/65) of the *LKB1/STK11* genomic regions by CISH analysis, jointly affecting ~88% of the tested tumors. Homozygous loss of both alleles (HD) for tumor suppressor genes is a result of a second allelic deletion of a chromosomal region that has undergone hemizygous loss in the other allele (Cairns *et al.*, 1995; Hahn *et al.*, 1996; Li *et al.*, 1997; Steck *et al.*, 1997). This high frequency of total loss we observed here (89% total) is consistent with the over 83% LOH in the 19p13.2 region observed in NSCLC cell lines (Virmani *et al.*, 1998), suggesting that the observed loss in NSCLC cell lines most likely originated from their primary tumors *in vivo*.

Several studies have investigated somatic mutations of the *LKB1/STK11* gene in various tumor types (Avizienyte *et al.*, 1998, 1999; Bignell *et al.*, 1998; Wang *et al.*, 1998; Guldborg *et al.*, 1999; Rowan *et al.*, 1999; Zhong *et al.*, 2006; Wingo *et al.*, 2009). Most studies indicate that *LKB1* is infrequently mutated in sporadic cancers except in adenocarcinoma of the lung (Carretero *et al.*, 2004) and more recently in cervical cancers of both adeno- and squamous cell pathologies (Wingo *et al.*, 2009). The majority of mutations in the gene result in loss of function. Matsumoto *et al.* (2007) analyzed 70 lung cancer cell lines, 106 primary adenocarcinomas and 25 brain metastases. In cell lines, point mutations and HD were observed in 13%(9) and 21%(13), respectively. In primary tumors, however, only one mutation was detected in primary adenocarcinomas and three were observed in brain metastases. In the present study, we observed a relatively low frequency of somatic mutations in the *LKB1* gene (11%). Among the tumors with a mutation in the *LKB1* gene, six were adenocarcinomas and one tumor was squamous cell carcinoma. All mutations were tumor specific and six out of the seven mutations occurred at novel mutation sites, except one nonsense case at codon 120 (E120X). In addition, our data revealed a racial difference in the loss of 19p13.2 with both copies lost in 35% of the Caucasians compared with 6% of the African Americans ( $P=0.04$ ). Although the sample size is too small in this subset to determine the robustness of the differences between *LKB1* gene changes in race and gender, a recent study by Koivunen *et al.* (2008) suggested strongly that ethnic background of the patient affects *LKB1* mutation frequency and spectrum among different patient populations. Cooperation between *LKB1* and K-ras was observed in using somatically activated K-ras-driven mouse model of lung cancer (Ji *et al.*, 2007). In this model, *LKB1*-deficient tumors had a short latency period. More recently, Ding *et al.* (2008) conducted a large resequencing effort and showed that mutation in *LKB1/STK11* gene was among the most common genes frequently mutated in lung adenocarcinoma (Guldborg *et al.*, 1999).

Unique to our study is the finding that a significant fraction of lung tumors harbor HD of the *LKB1* gene by direct CISH analyses. Our results demonstrated that the CISH method is highly sensitive in detecting the status of tumor samples for LOH and HD. Another advantage of CISH is that it is not limited by the informativeness of the microsatellite marker and could clearly be resolved under bright field microscopy on the basis of hybridization signals present in the tumor cell nuclei. In addition, the CISH method enabled us to directly assess the chromosomal copy number changes in primary lung cancer samples using TMA samples. In conclusion, our study provides the first direct evidence that 39% of primary NSCLC inactivate the *LKB1* gene by either HD or a mutation in the gene, and that nearly 90% of NSCLC has a loss of either single or both copies of the *LKB1/STK11* gene locus, highlighting its critical role in lung tumorigenesis process. Because small deletions below the detection limit of the CISH probe are unlikely to have been detected in our study, the actual frequency of chromosome loss for this region could approach 100%, making *LKB1* the highest site of inactivation in lung cancer.



## Materials and methods

### Tissue samples and DNA extraction

Tumor and matched non-tumor tissues from 124 patients diagnosed with primary lung cancer were obtained after surgical resection at the University of Maryland Medical Center. Samples were collected with informed consent, as approved by the respective institutional review boards. Representative sections from the tumor and paired non-tumor tissues were stained with hematoxylin and eosin and reviewed by a pathologist to determine the tumor histological subtype and to assess tumor content. Fresh frozen tissues from the tumors were grossly dissected to ensure that specimens contained at least 50% tumor cells. Approximately 10 sections of 20  $\mu\text{m}$  thickness were collected from non-tumor and tumor samples and placed in either Trizol reagents (Invitrogen, Carlsbad, CA, USA) for RNA or 1% SDS/proteinase K (10 mg/ml) solution at 58 °C overnight for DNA. For samples placed in Trizol, DNA was extracted from the organic phase after RNA extraction, following the manufacturer's protocol. All samples were extracted with phenol–chloroform and were ethanol precipitated. Clinical information was available from all 124 patients. The p53 mutation and staining analysis is described in Mechanic *et al.*, (2005).

### Microsatellite analysis

We screened for LOH at the short arm of chromosome 19 in 124 paired tumor/non-tumors. Eight fluorescent-labeled microsatellite markers located in chromosome 19p were obtained from Applied Biosystems (Foster City, CA, USA). These markers included D19S814, D19S883, D19S565, D19S1166, D19S424, D19S209, D19S894 and D19S884. For analysis of allelic deletions by LOH, polymerase chain reaction (PCR) was carried out using 6 ng of genomic DNA and 5 pmol of each primer (one of them cyanine 5-labeled). The true allele PCR premix (Applied Biosystems) was used for the PCR amplifications for 32 cycles of 94 °C for 15 s, 55 °C for 15 s, and 72 °C for 30 s, followed by incubation at 72 °C for 30 s. After the amplification, the PCR products were run on an ABI 3100 automatic sequencer together with LIZ 500 molecular weight markers and formamide (Applied Biosystems). Allelic loss was determined when the ratio was  $<0.75$  or  $>1.25$  for the informative alleles between the tumor and the paired non-tumor samples. The sample was considered as having an allelic imbalance when there were at least two markers demonstrating allelic imbalance among all eight chromosome 19p markers tested.

### CISH in lung TMA

**Identification and labeling of CISH probe**—CDT-2315C07, a BAC containing human chromosome regions identified via genetic analysis, was selected for the region around the *LBK1/STK11* gene (Invitrogen). This BAC is located at a genomic region 37 807 bp 3' to the *LKB1* gene, as shown in Figure 1a. DNA was isolated, labeled with biotin–UTP, and hybridized with normal blood lymphocyte metaphase-spread slides. The BAC CDT-2315C07 generated a strong and specific hybridization for the target region (data not shown) and was used as a probe for interphase nuclei CISH. For probe labeling and hybridization, conditions were the same as previously described (Gill *et al.*, 2008), and the probe was labeled with digoxigenin–UTP.

**CISH hybridization**—TMA slides containing tumor and normal samples were baked for 2–4 h at 60 °C before being processed for hybridization with a digoxigenin-labeled probe. Briefly, the tissue was deparaffinized and hydrated through an ethanol series, treated with 0.5mm dithiothreitol for 2 h at room temperature, followed by incubation in 1% sodium thiocyanate overnight at room temperature. After overnight incubation, the slides were incubated at 80 °C for 1 h. The tissues on the slides were further treated with Zymed pretreatment (tissue pretreatment kit; Invitrogen) for 45 min at 95–98 °C, digested with

protease solution from the tissue pretreatment kit (Invitrogen) for 14min, washed twice with 2× SSC, and allowed to dry at 37 °C. The tissue was then fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 10min at room temperature, washed twice in 2× SSC, and then denatured with 70% formamide and 2× SSC for 10min at 80 °C. Denatured slides were further digested sequentially with collagenase A 10 µg/ml (Roche, Basel, Switzerland) and proteinase K 10 µg/ml (Sigma, Milwaukee, WI, USA) in PBS at 37 °C for 15 and 13min, respectively. The slides were then blocked with avidin-blocking solution (DakoCytomation, Carpinteria, CA, USA) for 30min at room temperature and hybridized with preannealed probes for 36 h at 37 °C.

After the hybridization, the slides were washed in 50% formamide and 2× SSC at 45 °C three times for 5min each, 0.5× SSC and 0.1% SDS at 65 °C four times for 5min each, and 2× SSC at room temperature. After washing, they were incubated with casein blocking solution (Invitrogen) containing sheep or goat IgG (1:100 dilution) for 30 min at 37 °C to block nonspecific binding. After blocking, the slides were incubated with alkaline phosphatase-labeled sheep anti-digoxigenin antibody (Invitrogen) for 1 h at 37 °C. The slides were washed in 4× SSC 0.1% Tween-20 solution four times at 45 °C and then washed with 20× SSC twice at room temperature. The slides were developed with fast red substrate (DakoCytomation) for 1 h and then counter stained with Mayer's hematoxylin.

### Microscopy and CISH scoring

TMA slides were scanned by Aperio ScanScope GL System (Aperio, Vista, CA, USA) under an ×40 objective and viewed by Aperio ImageScope software (version 7.3.36; Aperio). Images of hybridization in lung tissue cores were manually scored for number of signals per tumor cell nuclei. Cores with weak signal intensities were analyzed using a Nikon E800 microscope under an ×60 objective. The signals were evaluated by first visually examining the entire core and then individually examining the tumor cell nuclei to determine the number of signals per cell. Nuclei unable to be evaluated due to insufficient hybridization or cell clustering were excluded from scoring. Approximately 100 nuclei were counted for each core. The number of signals per nuclei with the highest percentage (mode) in the sample was used to determine the genetic status of the LKB1 in the sample. A total of 65 samples were also analyzed by LOH study and included for comparisons.

### Genomic sequencing of lung tumors with LOH at chromosome

*19p*. We analyzed 62 of the 124 samples with LOH at markers D19S814 and/or D19S883 by direct sequencing. Genomic DNA from the tumor and matched non-tumor tissues were amplified using eight pairs of primers that amplified the entire coding region of the gene. The PCR reaction was performed using 10 ng of total genomic DNA and all exons of LKB1 were analyzed on an ABI PRISM 3730 (Applied Biosystems) automatic sequencer and subject to mutation analysis using the mutation detector of the Genome-work Bench (Zhang *et al.*, 2008). All exons of LKB1, including intron/exon borders, were directly sequenced in both directions. All alterations were confirmed to be somatic or germ line by sequencing the paired non-tumor samples.

### Statistical analysis

We compared the differences in the characteristics of lung cancer patients with or without LOH in *19p* and LKB1 mutations by *t*-test or analysis of variance for continuity, and  $\chi^2$ -tests or Fisher's exact tests (when 20% of the expected counts were less than 5) for categorical variables as indicated. We also performed comparisons of clinicopathological factors (that is, age at diagnosis, gender, histological type or pathological stage, and smoking status) in no LOH, with LOH and/or HD on the basis of CISH analysis. We used the log-rank test for comparing survival distributions between HD and LOH groups of LKB1/

STK11 loss, and we plotted Kaplan–Meier curves for the two groups. All statistical analyses were performed using SAS (SAS version 8.1; SAS Institute, Cary, NC, USA).

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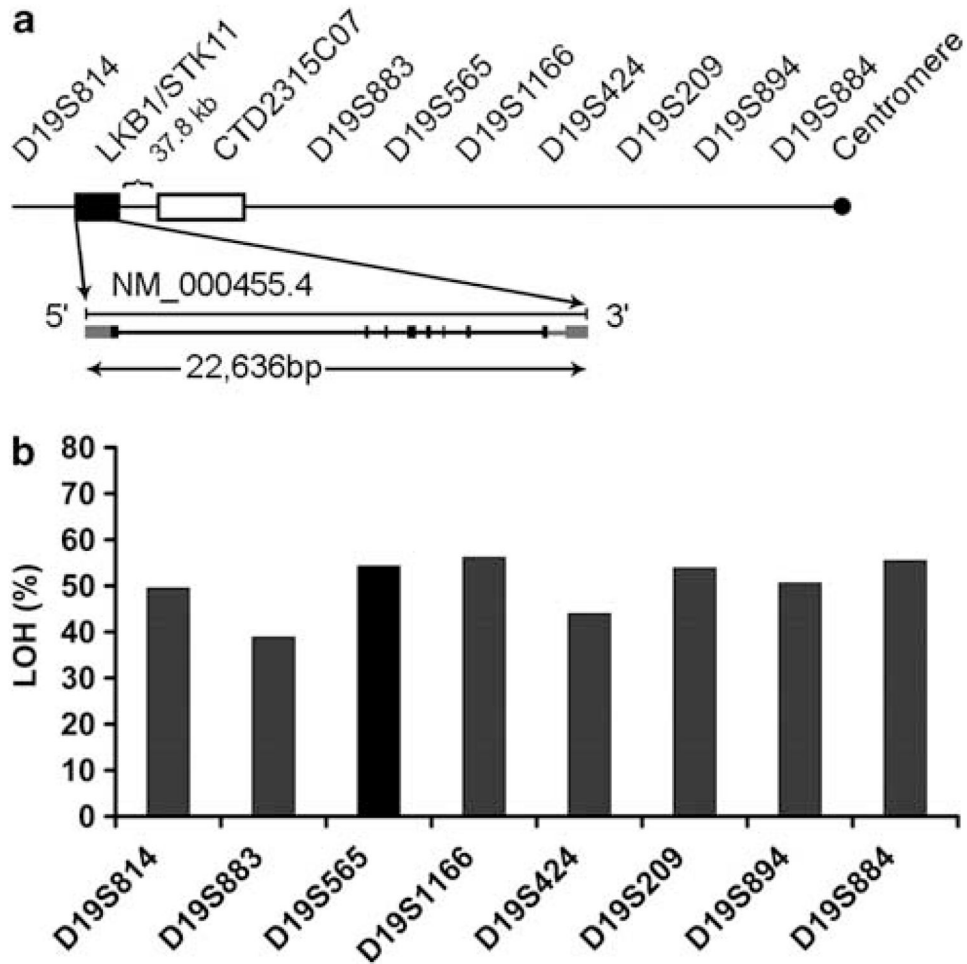
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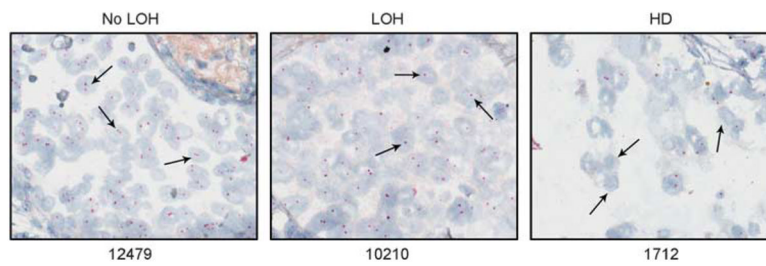
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**Figure 1.**

Loss of chromosome 19p and the LKB1/STK11 region in primary NSCLC (a) Schematic diagram of LKB1/STK11 locus in relation to CISH probe and tested microsatellite markers. Markers are listed from telomere (left) to centromere (right). The genomic organization of *LKB1/STK11* gene is shown (not to scale) and is 37.8 kb from the most 3' clone (CTD2315C07) available for CISH. (b) Frequency of LOH by microsatellite analysis. The marker D19S883 is the closest to the *LKB1* gene and had the lowest LOH frequency (38.8%) among all tested markers.



**Figure 2.**

Genomic status of the *LKB1/STK11* gene by CISH. Examples of hybridization signals for the *LKB1/STK11* gene in normal and tumor lung tissues. Arrows indicate the observed signals inside the cells. The prevailing (mode) distribution of the hybridization signals determines the genetic status of the gene in the tumor as no LOH (#12479), LOH (#10210) or HD (#1712).

Table 1

Correlation between LKB1/STK11 loss and clinicopathological characteristics of primary lung tumors

	Microsatellite analysis			LKB1-CISH			
	No LOH (n = 44) N (%)	LOH (n = 80) N (%)	P-value	No loss (n = 7) N (%)	LOH (n = 40) N (%)	HD (n = 18) N (%)	P-value
<i>Age (years ± s.d.)</i>	63.6 ± 11.3	65.2 ± 8.5	0.44 <sup>a</sup>	68.6 ± 8.9	64.2 ± 9.9	66.3 ± 7.0	0.93 <sup>b</sup>
37–65	20 (36.4)	35 (63.6)	0.85 <sup>c</sup>	2 (7.4)	20 (74.0)	5 (18.5)	0.24 <sup>d</sup>
66–84	23 (34.8)	45 (65.2)		5 (13.2)	20 (52.6)	13 (34.2)	
<i>Gender</i>							
Male	25 (31.7)	54 (68.3)	0.24 <sup>c</sup>	5 (12.2)	24 (58.4)	12 (29.3)	0.80 <sup>d</sup>
Female	19 (42.2)	26 (57.8)		2 (8.3)	16 (66.7)	6 (25)	
<i>Race</i>							
African American	12 (30.8)	27 (69.2)	0.45 <sup>c</sup>	3 (17.7)	13 (76.5)	1 (5.9)	0.04 <sup>d</sup>
Caucasian	32 (37.6)	53 (62.4)		4 (8.3)	27 (56.3)	17 (35.4)	
<i>Smoking status</i>							
Never	9 (56.3)	7 (43.7)	0.11 <sup>c</sup>	0 (0)	2 (66.7)	1 (33.3)	0.71 <sup>d</sup>
Former	15 (35.9)	26 (63.4)		4 (17.4)	12 (52.2)	7 (30.4)	
Current	19 (28.8)	47 (71.2)		3 (7.7)	26 (66.7)	10 (25.6)	
<i>Pack years</i>							
2.5–45	12 (33.3)	24 (66.7)	0.94 <sup>c</sup>	2 (10.0)	12 (60.0)	6 (30.0)	0.47 <sup>d</sup>
46–68	10 (29.4)	24 (70.6)		2 (10.0)	10 (50.0)	8 (40.0)	
70–158	11 (30.6)	25 (69.4)		3 (14.3)	15 (71.4)	3 (14.3)	
<i>Stage</i>							
I	22 (28.6)	55 (71.4)	0.04 <sup>c</sup>	4 (10.3)	25 (64.1)	10 (25.6)	0.93 <sup>d</sup>
II–IV	22 (46.8)	25 (53.1)		3 (11.5)	15 (57.7)	8 (30.8)	
<i>Pathology</i>							
Adenocarcinoma	20 (29.4)	48 (70.6)	0.36 <sup>c</sup>	2 (5.7)	22 (62.9)	11 (31.4)	0.34 <sup>d</sup>
Squamous cell	19 (38.8)	30 (61.2)		5 (17.2)	17 (58.6)	7 (24.1)	
<i>p53 mutation</i>							
+ Mutation	28 (37.8)	47 (62.7)	0.17 <sup>c</sup>	4 (8.5)	31 (66.0)	12 (25.5)	0.31 <sup>d</sup>
– Mutation	5 (21.7)	18 (78.3)		3 (17.7)	8 (47.1)	6 (35.3)	
<i>p53 staining</i>							
Negative	24 (36.4)	42 (63.6)	0.42 <sup>c</sup>	4 (9.1)	25 (56.8)	15 (34.1)	0.29 <sup>d</sup>
Positive	9 (28.1)	23 (71.9)		3 (15.0)	14 (70.0)	3 (15.0)	

Abbreviations: CISH, chromogenic *in situ* hybridization; HD, homozygous deletion; LOH, loss of heterozygosity.<sup>a</sup> *t*-test for unequal variances.<sup>b</sup> Analysis of variance<sup>c</sup>  $\chi^2$ -test.<sup>d</sup> Fisher's exact test.The boxes illustrate comparisons with significant *P*-value.



**Table 2**

CISH signal distribution in all tested normal and tumor lung tissue samples

Tissue type	Signals/cell (%)				
	0	1	2	3	4
<i>Normal</i>					
Non-tumor (n=9)	3.6	10.9	<b>80</b>	4.1	1.3
Adjacent normal (n=8)	4.5	20	<b>73.5</b>	1.9	0.1
<i>Tumor</i>					
No LOH (n=7)	9.1	24	<b>51.9</b>	9.8	5.3
LOH (n=40)	19.2	<b>46.7</b>	28.1	4.4	1.5
HD (n=18)	<b>53</b>	33.4	12.2	0.6	0

Abbreviations: CISH, chromogenic *in situ* hybridization; HD, homozygous deletion; LOH, loss of heterozygosity.

The most prevalent mode of chromosome copy at indicated numbers are shown in bold.

Table 3

## Association of LOH markers with CISH

CISH	D19S883			D19S814			Both markers		
	Normal (n = 34) N (%)	LOH (n = 22) N (%)	P-value	Normal (n = 25) N (%)	LOH (n = 26) N (%)	P-value	Normal (n = 17) N (%)	LOH (n = 27) N (%)	P-value
Normal	6 (18)	1 (5)	<0.0001 <sup>a</sup>	5 (20)	0 (0)	0.003 <sup>a</sup>	5 (29)	0 (0)	0.0006 <sup>a</sup>
LOH	13 (38)	21 (95)		11 (44)	22 (85)		6 (35)	23 (85)	
HD	15 (44)	0 (0)		9 (36)	4 (15)		6 (35)	4 (15)	

Abbreviations: CISH, chromogenic *in situ* hybridization; HD, homozygous deletion; LOH, loss of heterozygosity.

<sup>a</sup>Fisher's exact test.

**Table 4**

Characteristics of non-small cell lung patients with LKB1 gene mutations<sup>a</sup>

Sample <sup>b</sup>	Ethnicity	Gender	Age	Smoking	Pathology	Stage	Family Hx	Mutation	Exon	Nucleotides/amino acid change
1024T	AA	Female	63	CS (26PY)	ADC	III	No	Point mutation	2	Glu (GAA) 120 stop (TAA)
11669T	C	Female	67	FS (105PY)	ADC	I	Yes	Point mutation	3	Gln (CAG) 152 stop (TAG)
10152T	AA	Female	61	CS (21PY)	ADC	III	No	Point mutation	4	Gly (GGG) 180 Val (GTG)
10403T	AA	Female	55	CS (84PY)	ADC	I	No	1-bp Deletion	5	Asp (GAC) 237 frameshift (-AC)
1571T	C	Female	70	CS (50PY)	SCC	I	Yes	1-bp Deletion	6	Pro (CCG) 281 frameshift (C-G)
11807T	AA	Female	73	FS (35PY)	ADC	I	Yes	Point mutation	7	Arg (AGG) 297 Ser (AGC)
10272T	C	Female	70	FS (33PY)	ADC	IV	Yes	Point mutation	7	Arg (CGG) 304 Gly (GGG)

Abbreviations: AA, African American; ADC, adenocarcinoma; C, Caucasian; CS, current smoker; FS, former smoker; NS, non-smoker; PY, pack/day years; SCC, squamous cell carcinoma.

<sup>a</sup>Reference gene ID: NM 000455.

<sup>b</sup>Known mutation site