Oncogene (2004) 23, 4037–4040

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Novel and natural knockout lung cancer cell lines for the *LKB1/STK11* tumor suppressor gene

Julian Carretero[1], Pedro P Medina[1], Ruben Pio[2,4], Luis M Montuenga[3,4] and Montserrat Sanchez-Cespedes*[,1]

[1]*Lymphoma and Lung Cancer Group, Molecular Pathology Program, Centro Nacional de Investigaciones Oncologicas (CNIO), Calle Melchor Fernández Almagro, 3, 28029 Madrid, Spain;* [2]*Department of Biochemistry, Foundation for Applied Medical Research, School of Medicine, University of Navarra, 31080 Pamplona, Spain;* [3]*Department of Histology and Pathology, Foundation for Applied Medical Research, School of Medicine, University of Navarra, 31080 Pamplona, Spain;* [4]*Division of Oncology, Foundation for Applied Medical Research, School of Medicine, University of Navarra, 31080 Pamplona, Spain*

Germline mutations of the *LKB1* gene are responsible for Peutz–Jeghers syndrome (PJS), an autosomal dominant inherited disorder bestowing an increased risk of cancer. We have recently demonstrated that *LKB1* inactivating mutations are not confined to PJS, but also appear in lung adenocarcinomas of sporadic origin, including primary tumors and lung cancer cell lines. To accurately determine the frequency of inactivating *LKB1* gene mutations in lung tumors we have sequenced the complete coding region of *LKB1* in 21 additional lung cancer cell lines. Here we describe the mutational status of *LKB1* gene in 30 lung cancer cell lines from different histopathological types, including 11 lung adenocarcinomas (LADs) and 11 small cell lung cancers (SCLCs). *LKB1* gene alterations were present in six (54%) of the LAD cell lines tested but in none of the other histological types. Similar to our previous observations in primary tumors, all point mutations were of the nonsense or frameshift type, leading to an abnormal, truncated protein. Moreover, 2 cell lines (A427 and H2126) harbored large gene deletions that spanned several exons. Hence, we have identified additional lung cancer cell lines carrying inactivating mutations of the *LKB1* tumor suppressor gene, further attesting to the significance of this gene in the development of LADs and providing new natural *LKB1* knockouts for studies of the biological function of the LKB1 protein.
Oncogene (2004) **23,** 4037–4040. doi:10.1038/sj.onc.1207502
Published online 15 March 2004

Keywords: LKB1/STK11; Peutz–Jeghers syndrome; lung adenocarcinomas

Introduction

LKB1, located on chromosome 19p, is the tumor suppressor gene involved in the autosomal dominant, cancer-predisposing disorder called Peutz–Jeghers syndrome (PJS) (Hemminki *et al*., 1998; Jenne *et al*., 1998). Commonly, the *LKB1* germline alterations detected in patients with PJS generate premature truncated proteins, either by nonsense or frameshift mutations in the coding sequence or by partial or complete deletions of the gene (Hemminki *et al*., 1998; Jenne *et al*., 1998). The LKB1 protein is a serine/threonine kinase, and among its biological substrates are PAR1 (Spicer *et al*., 2003), which is required for establishing cell polarity during embryogenesis, and AMPK (Hawley *et al*., 2003; Woods *et al*., 2003), a key regulator of cellular metabolism. In spite of its relevance in tumor development it is not yet completely understood how *LKB1* gene inactivation contributes to tumor development. Recent reports show that overexpression of LKB1 leads to cell growth inhibition in certain cancer cells due to p21-induced cell cycle arrest during G1 (Tiainen *et al*., 1999), to growth arrest induced by the association with Brg1 (a component of the chromatin remodeling complexes) (Marignani *et al*., 2001), or to apoptosis through its physical interaction with TP53 (Karuman *et al*., 2001). Moreover, we have recently reported that ectopic expression of LKB1 in deficient cells leads to an increase in the expression of several TP53-responsive genes and of PTEN (Jimenez *et al*., 2003). Finally, numerous proteins such as LIP1, STRADα and MO25α/β have been reported to interact and regulate LKB1 activity (Smith *et al*., 2001; Baas *et al*., 2003; Boudeau *et al*., 2003).

During the past decade, considerable effort has been dedicated to the search for new tumor suppressor genes in sporadic tumors. Genome-wide screening using LOH, CGH and other approaches has identified several chromosomal arms that are frequently lost in cancer. However, in most cases, the targeted tumor suppressor gene remains elusive (Sanchez-Cespedes, 2003). In lung cancer, one of the most frequently deleted chromosomal arms is the short arm of chromosome 19 (Virmani *et al*., 1998; Sanchez-Cespedes *et al*., 2001). We have previously reported that *LKB1* is the tumor suppressor gene targeted in this region, because inactivating somatic *LKB1* gene mutations were present in one-third of

*Correspondence: M Sanchez-Cespedes; E-mail: msanchez@cnio.es
Received 8 January 2004; revised 12 January 2004; accepted 12 January 2004; Published online 15 March 2004

sporadic lung adenocarcinomas (LADs) tested, including 32 primary tumors and nine lung cancer cell lines (Sanchez-Cespedes *et al*., 2002).

To determine the precise frequency of *LKB1* gene inactivation in lung tumors, avoiding the confounding influence of normal cell contamination that often masks the presence of partial deletions, and to identify possible differences in the *LKB1* mutation pattern among distinct lung cancer histologies, we have now screened 21 additional lung cancer cell lines for *LKB1* gene alterations. We present data from the mutational analysis in a panel of 30 lung carcinomas including 11 LADs, 11 small cell lung cancers (SCLCs) and others (Table 1).

Our results revealed the presence of novel *LKB1* gene alterations in cell lines H1395, A427, H2126 and H460

Table 1 Lung cancer cells lines tested for *LKB1* gene alterations. Asterisks indicate those cells that were screened in our previous report (Sanchez-Cespedes *et al*., 2002)

SCLC	*NSCLC*			*Others*
	LAD	*SCC*	*Others*	
H69	A427	SK-MES-1	H661	H727
H82	A549*	H226	H1155*	
H187	Calu-3		H1299*	
H209*	H23*		U1752	
H345	H358*		H1770*	
H446	H441			
H510	H460			
H1618*	H522*			
N417	H1395			
H249	H2087			
H774	H2126			

Table 2 *LKB1* gene alterations in lung adenocarcinoma cell lines. Mutational data for *KRAS* and *TP53* genes were obtained from the NCI tumor cell line database (http://www.atcc.org/SearchCatalogs/ncisearch.cfm) or by direct sequencing of PCR products using standard protocols

Cell line	*Mutation type (codon)*	*Predicted product*	*Other mutations*
A427	Exons 1–5	Deletion, absence	*KRAS*
A549[a]	cag-tag (37)	Gln to Stop	*KRAS*
H460	cag-tag (37)	Gln to Stop	*KRAS*
H1395	ctgggggaa to ctggggaa (56)	Deletion, frameshift	*BRAF1*[b]
H23[a]	tgg-tga (332)	Trp to Stop	*TP53, KRAS*
H2126	Exons 4–6	Deletion, absence	*TP53*

[a]*LKB1* mutations at these cell lines have previously been reported by Sanchez-Cespedes *et al*., 2002. [b]*BRAF1* mutation in the H1395 cells has previously been reported by Davies *et al*., 2002

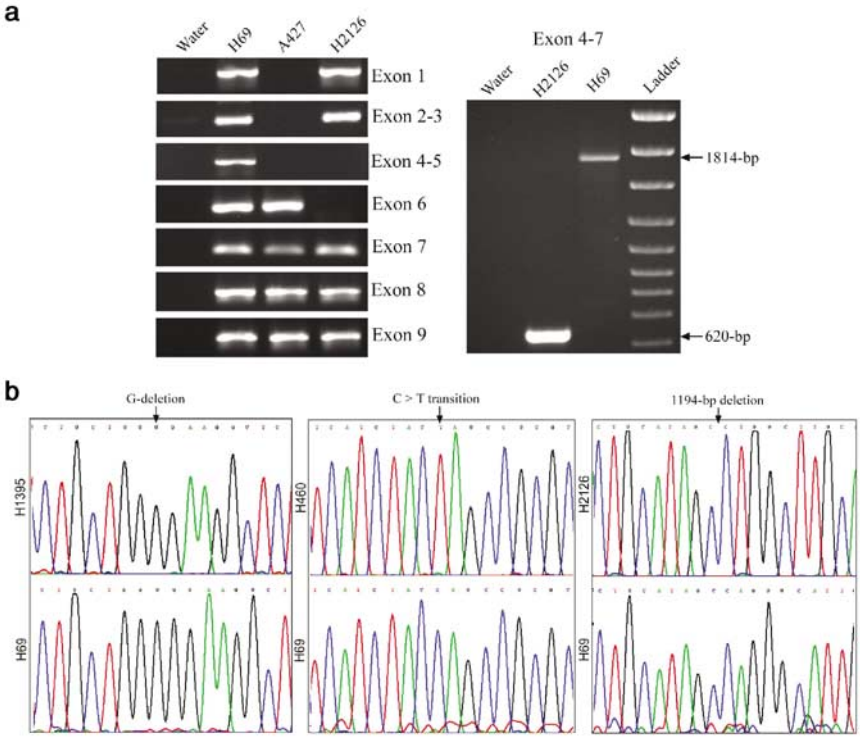

Figure 1 Genetic alterations of *LKB1* in lung cancer cell lines. DNA from cell lines was extracted following standard protocols. To screen for *LKB1* gene mutations, we used 30–50 ng of genomic DNA for exon amplification. Cycle sequencing reaction was performed according to the manufacturer's protocol (Perkin-Elmer, Roche Molecular Systems). Products were automatically sequenced in an ABI PRISM 3700 DNA Analyser (PE Biosystems). Details of PCR conditions and primers used have been reported elsewhere (Sanchez-Cespedes *et al*., 2002). (**a**) On the left, PCR products of indicated exons show absence of amplification, indicating deletions at some exons of the *LKB1* gene in the A427 and the H2126 cell lines. Appropriate negative and positive (H69 cells) controls are also included. The 1194-bp deletion in the H2126 cells can be observed on the right. (**b**) Electropherograms showing a 1-bp deletion in codon 56 at the H1395 cells, a nonsense mutation at codon 37 (Gln-stop) at the H460 cells and the junction of the deletion at the H2126 cells. Normal controls are also included

(Table 2 and Figure 1). The H1395 cells carry a 1-bp deletion in a stretch of guanines at codon 56 (exon 1); the A427 cells have a partial deletion of the gene, spanning from exons 1 to 5; the H460 cells have a C-to-T mutation at codon 37, leading to a change from Gln to a stop codon; the H2126 cells have a 1194-bp deletion that abrogates exons 4–6. The latter observation was confirmed by RT–PCR, as shown in Figure 2. As can be seen in Figure 1, all *LKB1* gene alterations were homozygous, indicating the loss of the remaining allele. Intriguingly, the nonsense mutation at codon 37 in the H460 cells is identical to that we previously reported for the A549 cells and for a primary tumor (Sanchez-Cespedes *et al*., 2002). We ruled out the possibility of cross-contamination among cell lines because two common germline polymorphisms at introns 1 and 3 were different in the A549 and H460 cells. A detailed search of the mutational databases for *LKB1* revealed that this point mutation has never been reported in PJS patients or in other sporadic tumors, implying that there is a mutational hot spot in LADs. Among the *LKB1* mutations closest to this putative hotspot reported to date there is a somatic nonsense mutation at codon 36 in a pancreatic tumor (Su *et al*., 1999) and a frameshift mutation at codon 37 in a PJS family (Miyaki *et al*., 2000). Mutational hot spots are fairly common in tumor suppressor genes. It is well known that mutations at guanine positions in codons 157, 248 and 273 at the *TP53* gene are hallmarks of lung tumorigenesis, which has been associated with exposure to tobacco carcinogens (see Hainaut and Pfeifer, 2001 for review).

Overall, of the 11 LADs cell lines that we have tested to date, six (54%) carry *LKB1* gene alterations, all predicting completely aberrant proteins (Table 2). Such a high frequency is only comparable to the rates reported for *TP53* mutations, indicating that *LKB1* is among the most important tumor suppressor genes in LADs. In our previous report (Sanchez-Cespedes *et al*., 2002), *LKB1* mutations were detected in one-third of primary LADs. The slight discrepancy between the rates of *LKB1* gene inactivation in primary LADs and in lung cancer cell lines probably implies that *LKB1* gene alteration frequencies are underestimated in primary tumors. In DNA extracted from primary tumors, the masking effect of contaminant normal cells that hinders the detection of large deletions and complex gene rearrangements is well known. Accordingly, the *LKB1* gene deletions observed in the A427 and H2126 cell lines would have remained undetected in a primary tumor.

Another definitive observation is the clustering of *LKB1* mutations in specific lung tumor histology. Whereas half of the LADs carry *LKB1* gene mutations, none of the 11 SCLC cell lines evaluated in the present study nor any of the 12 primary SCCs tested in our previous work (Sanchez-Cespedes *et al*., 2002) revealed *LKB1* gene alterations. These observations are in agreement with the significantly decreased frequency of LOH at chromosome 19p in SCLCs in comparison with NSCLC (Virmani *et al*., 1998). Most of the malignant tumors that arise in PJS patients are adenocarcinomas (Giardiello *et al*., 1987), suggesting that *LKB1* inactivation may be involved in the development of this specific tumor histology. Since it is possible that other components of the LKB1 biological pathway contribute to the development of lung tumors devoid of *LKB1* gene alterations, increased effort should be made to elucidate the biological function of LKB1 and to identify all LKB1 substrates, since these are likely targets for gene mutations.

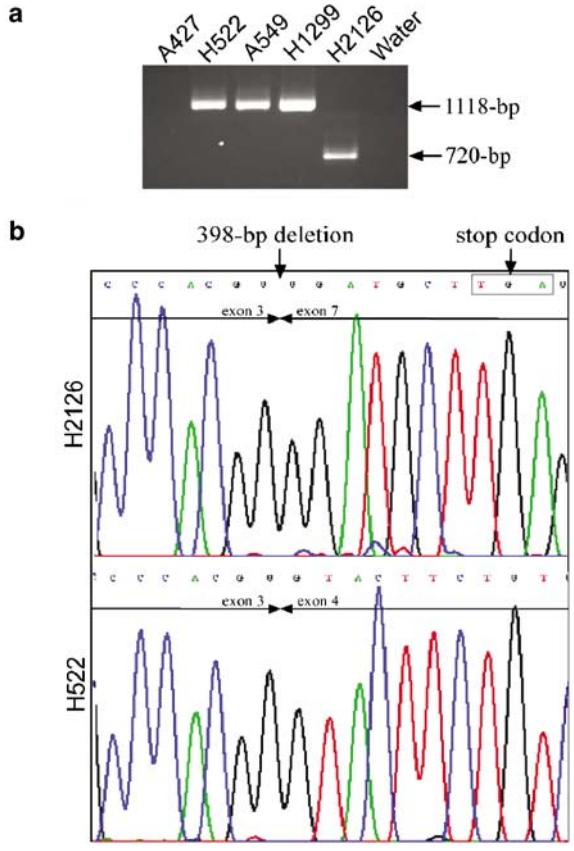

Figure 2 RT–PCR analysis of LKB1 mRNA in some of the lung cancer cell lines. Total RNA was extracted following the manufacturer's instructions (RNeasy kit, Quiagen). A measure of 2 μg of total RNA were reverse-transcribed with the Access RT System (Promega) using random hexamers and 2 μl of produced cDNA were amplified by standard protocols with specific primers for the 1.2-kb fragment of the LKB1 cDNA. (**a**) RT–PCR of the complete LKB1 transcript. The absence of RT–PCR product for the A427 cells and a shorter product in the H2126 cells (398-bp deletion) can be observed. (**b**) sequence of the cDNA indicating the exact junction point between exons 4 and 7 that generates a premature stop codon, as indicated. The wild-type sequence for the H522 cells is also included as normal control

Finally, we sought to identify a possible pattern of *LKB1* mutations linked to tobacco carcinogens. We compared the eight nucleotide change mutations described to date in sporadic LADs (Avizienyte *et al*., 1999; Sanchez-Cespedes *et al*., 2002 and present work) with the mutations in PJS patients (http://archive.uwcm.ac.uk/uwcm/mg/search/9732383.html). Although the number of *LKB1* mutations in the sporadic lung tumors is still too small to reach definitive conclusions, no significant differences were observed between the two distinct populations of tumors. G→T or C→A

transversions are more frequent in the *p53* and *KRAS* gene mutations that arise in sporadic lung tumors from smokers as compared to nonsmoking-related tumors (Ahrendt *et al*., 2001; Pfeifer *et al*., 2002). In our analysis, 29 and 25% of the nucleotide changes at the *LKB1* gene were G→T/C→A transversions in sporadic LADs and in tumors from PJS patients, respectively. The frequency of C→T/G→A transitions was also similar between the two groups, 50 and 35% in sporadic LADs and in tumors from PJS patients, respectively.

To date, only a few cancer cell lines lacking wild-type LKB1 protein have been reported: the melanoma cells G361 (Tiainen *et al*., 1999), the cervical adenocarcinoma cells HeLa S3 (Tiainen *et al*., 1999), and the lung adenocarcinoma cells A549 and H23 (Sanchez-Cespedes *et al*., 2002). Here, we report the presence of four additional lung cancer cell lines carrying inactivating *LKB1* gene alterations, thus identifying new natural 'knockout cell lines' with distinct *KRAS,* and *TP53* genetic background (Table 2). These observations provide new tools for studies of the biological function of the LKB1 protein. For example, recent evidence from HeLa cells and from *Lkb1*-knockout mouse embryonic fibroblasts indicates that LKB1 is the upstream-activating kinase for the stress-responsive AMP-activated kinase (AMPK) (Hawley *et al*., 2003). Our present results identify new *LKB1*-deficient cells that should verify these observations in other tissues such as lung.

In conclusion, we have shown that *LKB1* gene inactivation is present in half of the LAD cells tested, thereby demonstrating its relevance in the carcinogenesis of this tumor type and providing additional tools for unraveling the biological function of LKB1.

Acknowledgements

The research was supported by a grant from the Spanish Ministerio de Ciencia y Tecnología (SAF2002-01595) and by a grant from the Comunidad de Madrid (CAM 08.1/0032/2003 1). Julian Carretero is a postdoctoral fellow of the Excmo, Ayuntamiento de Madrid, Pedro P Medina is supported by a Comunidad Autonoma de Madrid fellowship and Montserrat Sanchez-Cespedes is supported by the Ramon y Cajal Program from the Ministerio de Ciencia y Tecnología, Spain.

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