Expression profiling and microRNA regulation of the LKB1 pathway in young and aged lung adenocarcinoma patients

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Abstract. Lung cancer in young patients appears to have distinct clinicopathological features. The present study focused on the role of the serine/threonine kinase liver kinase B1 (*LKB1*), a known tumor suppressor gene, and its miRNA regulation in lung adenocarcinoma, particularly in young versus elderly patients. A total of 88 patients with lung adenocarcinoma were retrospectively analysed. A simultaneous quantification was performed of the expression of *LKB1* mRNA and 15 microRNAs (miRNA/miRs; miRs -93, -96, -34a, -34c, -214, -33a, -30b, -145, -182, -30c, -183, -29b, -29c, -153 and -138) involved in the LKB1 pathway, as well as of 5 identified target mRNAs [cyclin D1 (*CCND1*), catenin β-1 (*CTNNB1*), lysyl oxidase (*LOX*), yes-associated protein 1 (*YAP1*) and survivin], using NanoString technology. *KRAS* mutations were investigated by pyrosequencing analysis. Patients ≤50 years were defined as a younger group, while patients >50 years old as an older group (n=44/group). No difference between the two groups was identified in terms of survival times analysed using the Kaplan-Meier method or *KRAS* mutations. Subsequently, the LKB1 signalling pathway was focused on, as a target for therapy in lung adenocarcinoma, and assessed with regards to clinicopathological features; we found that *LOX* levels in adenocarcinoma patients were significantly associated with histological subtype (P=0.03), stage (P<0.0001) and prognosis (P=0.02 for disease-free interval and P=0.005 for overall survival), but not with age. Furthermore, the miRNA target prediction model indicated that miR-93 and miR-30b appeared to have functional binding sites and downregulate the gene expression of *LKB1* and *LOX*, respectively. In conclusion, young patients appeared have similar survival rates to elderly patients. The assessment of *LKB1*, its downstream genes and its regulation by miRNAs may have an impact on future research on lung adenocarcinoma in young and elderly patients. Further investigations will be necessary to elucidate the potential of this pathway as a novel target for therapy.

Introduction

Lung cancer remains the main cause of cancer-related deaths worldwide (1). Cases of non-small cell lung cancer (NSCLC) in young patients (≤50 years old) represent a small percentage of the total cases, and indeed this disease typically affects older individuals (>50 years old), and the incidence rate among elderly patients is increasing (2). Previous studies have compared young and aged NSCLC patients, using the range of 40 to 50 years to define the young group (2-8); however, to date, it is not clear if lung cancer, particularly adenocarcinoma, in young patients may have distinct clinicopathological features. In the present paper, liver kinase B1 (*LKB1*) and its downstream signalling pathways were investigated as a therapeutic target in lung adenocarcinoma, a subtype of NSCLC (9), and compared between different age groups. *LKB1*, also known as serine/threonine kinase 11, is a tumor suppressor gene involved in cellular responses including growth, polarity and metabolism (10). LKB1 is a master kinase, controlling 14 substrates involved in the translation of several cell growth regulators (11). *LKB1* was been initially identified as the tumor suppressor responsible for Peutz-Jeghers syndrome, an inherited cancer predisposition (12). Several sporadic tumors exhibited *LKB1* promoter hypermethylation, reduced *LKB1* expression and somatic LKB1 mutations, indicating a role of the loss of LKB1 in cancer development and progression, potentially with additional oncogenic factors (13). *LKB1* may also be repressed as a result of post-transcriptional regulation by microRNAs (miRNA/miRs) (10), which generally serve to repress mRNA translation or promote mRNA degradation via partial complementary binding to the 3' untranslated region (3'-UTR) of target mRNAs (14).

The role of LKB1 in NSCLC has previously been analysed; however, study is made difficult by the fact that the LKB1 pathway involves multiple substrates that act on metabolism, apoptosis and the tumor microenvironment. Carretero *et al* (15) reported that NSCLC cells with loss of LKB1 exhibited higher nuclear expression of catenin β-1 (CTNNB1); LKB1, in fact, appears to suppress the Wnt/CTNNB1 pathway, inhibiting the expression of downstream genes, including cyclin D1 (*CCND1*) and *survivin*. Additionally, lysyl oxidase (LOX) has been reported to be negatively regulated by LKB1 in

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lung cancer, and yes-associated protein 1 (YAP1) has been reported to be activated in lung adenocarcinoma as a result of a lack of LKB1 (16). In the present study, the mRNA expression of *LKB1*, *CCND1*, *CTNNB1*, *LOX*, *YAP1*, *survivin* and 15 miRNAs involved in the *LKB1* pathway was investigated using NanoString technology. *LKB1* loss has been reported to be more common within *KRAS*-mutant lung adenocarcinomas (17), and therefore, *KRAS* mutations were also investigated.

The results presented provide indication that *LKB1* pathway genes, with the involvement of miRNA regulation, may have a role in lung adenocarcinoma progression, representing novel potential targets for lung cancer therapy.

Patients and methods

Patients. A total of 88 lung adenocarcinoma patients were retrospectively selected from patients who were operated between January 2003 and December 2013 at the Unit of Thoracic Surgery of the University Hospital of Pisa (Pisa, Italy). Histological diagnoses were made according to the World Health Organization classification (9,18,19). Data on clinicopathological characteristics were collected for all patients (Table I). The study was conducted in accordance with the 1964 Helsinki declaration and the ethical standards of Institutional Research Committee of the University of Pisa, for the collection of lung cancer samples following surgery and the related informed consensus for molecular analysis. Patients ≤50 years old were defined as the younger group (n=44), and patients >50 years old as the older group (n=44).

Target prediction. A total of 15 miRNAs (miRs -93, -96, -34a, -34c, -214, -33a, -30b, -145, -182, -30c, -183, -29b, -29c, -153 and -138) were selected based on their involvement in the LKB1 pathway (20-29). Alignment of miRNAs with target genes (*LKB1*, *CCND1*, *CTNNB1*, *LOX*, *YAP1* and *survivin*) was predicted by using the microRNA target prediction program (http://www.microrna.org).

DNA and RNA isolation. DNA, RNA and miRNAs were isolated from 5-10 *µ*m sections of formalin-fixed (buffered formalin, for 24-48 h at room temperature) and paraffin-embedded (FFPE) resected tissues, performed immediately following surgery, following manual tumor macrodissection using a QIAamp DNA Mini kit (Qiagen GmbH, Hilden, Germany) and a miRNeasy FFPE kit (Qiagen GmbH), respectively, according to the manufacturer's instructions. The quality and concentration were assessed using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Inc., Waltham, MA, USA).

NanoString nCounter® assay, data normalization and analysis. Expression of the 6 targeted mRNAs and 15 selected miRNAs was measured using the NanoString nCounter Technology system, according to the manufacturer's protocol (NanoString Technologies, Inc., Seattle, WA, USA). The nCounter measures the total counts of mRNAs/miRNAs through a multiplex hybridization assay, followed by scanning and digital readout of fluorescent probes in a high-throughput manner (30). The nCounter custom code set used in the current study included the 6 targeted genes and 3 housekeeping genes

Table I. Lung adenocarcinoma patient characteristics in the two groups.

Variable	Young	Old	Total	P-value
Sample size	44	44	88	-
Age, years	46.3±3.9	71.5±5.2	58.9±13.4	-
Sex				0.02
Male	23	33	56	
Female	21	11	32	
Adenocarcinoma prevalent pattern				0.0004
Lepidic	13	16	29	
Solid	9	17	26	
Acinar	19	3	22	
Papillar	3	8	11	
Tumour grading				0.07
G1	3	0	3	
G2	30	28	58	
G3	11	16	27	
Stage				0.79
IA	8	9	17	
IB	14	9	23	
IIA	6	7	13	
IIB	4	5	9	
IIIA	10	13	23	
IIIB	1	0	1	
IV	1	1	2	
KRAS status				0.07
Wild-type	31	23	54	
Mutant	13	21	34	

Age is provided as the mean ± standard deviation; all other values represent case number.

as references (tubulin β, hypoxanthine phosphoribosyltransferase and phosphoglycerate kinase 1). Raw NanoString counts for each gene were subjected to technical and biological normalization using the positive control probe sets and three reference genes, respectively. miRNAs were normalized using a scaling factor based on the 5 miRNAs with the lowest variability coefficients according to the manufacturer's protocol.

KRAS mutation analysis. Pyrosequencing analysis was performed using the PyroMark Q96 ID platform (Diatech Pharmacogenetics SRL, Jesi, Italy) following the manufacturer's instructions in order to determine KRAS status. Codons 12, 13, 61, 117 and 146 of the KRAS gene were analysed.

Statistical analysis. The normalized RNA hybridization data, presented as direct counts of digital reports, were analysed by using nSolver 2.5 analysis software (NanoString Technologies, Inc.). The χ^2 test was applied to analyze lung adenocarcinoma patient characteristics in the two age groups and to determine the association between *LKB1* and miR-93 expression.
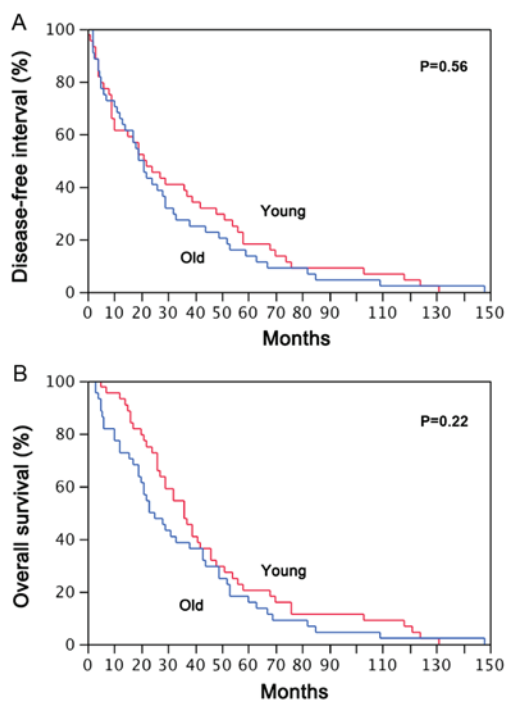
Figure 1. Kaplan-Meier curves for the analysis of (A) disease-free interval and (B) overall survival in younger and older lung adenocarcinoma patients (n=44/group).

Differential gene expression was determined by applying the non-parametric t test and analysis of variance. Survival analyses were performed using the Kaplan-Meier method with the log-rank test and the Cox proportional hazard model. Statistical analyses were performed using JMP 10 software (SAS Institute, Inc., Cary, NC, USA), and two-tailed P<0.05 was considered to indicate statistical significance.

Results

Comparison of patient characteristics between the age groups. The current study was conducted in 88 patients with lung adenocarcinoma (56 males and 32 females). Patients ≤50 years old were defined as the younger group, and patients >50 years old as the older group. Among all patients, different histological subtypes of adenocarcinoma were identified; the most common histological subtypes were lepidic (29/88, 33.0%), solid (26/88, 29.5%), acinar (22/88, 25.0%), and papillar (11/88, 12.5%). The median age at diagnosis was 54.5 years old (range, 30-81 years; mean, 58.9±13.4 years). Regarding grading, 3 tumors (3.4%) were G1, whereas 58 (65.9%) and 27 (30.7%) were G2 and G3, respectively. The adenocarcinomas were all invasive, and stages 17 IA, 23 IB, 13 IIA, 9 IIB, 23 IIIA, 1 IIIB, and 2 IV were identified, according to the World Health Organization classification (9,18,19). The follow-up data, disease-free interval (DFI) and overall survival (OS) were available for all patients and were last updated on March 2015. Disease progression (recurrence/metastasis) was observed in 50 patients (56.8%; data not shown). Regarding smoking habits, there were 17 non-smokers, 16 former smokers and 23 current smokers; for 33 patients, the smoking data were not available. Regarding clinicopathological characteristics, overall gender distribution (P=0.02) as well as histological subtype distribution (P=0.0004) were significantly different between the younger and older cases (Table I).

Comparison of survival between the age groups. The median DFI and OS times of total patients (n=88) were 21 months (range, 0-148) and 31.5 months (range, 3-148), respectively.

The median DFI times were 22 months in younger patients and 21 months in older patients; the median OS times were 36 and 23 months in the younger and older groups, respectively. Survival analysis using the Kaplan-Meier method with DFI and OS as endpoints did not identify a significant difference between younger patients and their elderly counterparts (Fig. 1).

LKB1 pathway expression. To investigate the role of the LKB1 pathway in lung adenocarcinoma, first the levels of LKB1 were screened. As presented in Table II, there was no significant association of *LKB1* expression with patient age, prevalent adenocarcinoma pattern or tumor grading; however, the data indicated a significant association of low *LKB1* expression with male gender (P=0.03) and overall clinical stage (P=0.01) as well as a trend with the solid variant. Next it was investigated whether the expression of downstream genes and their regulation was directly affected by *LKB1* levels. Low *LKB1* expression was associated with low expression of *CCND1* (P<0.0001), *CTNNB1* (P<0.0001) and *YAP1* (P=0.0024; data not shown), suggesting regulation by *LKB1*. To test if *LOX*, one of the other *LKB1* network partners, was an important downstream mediator of lung adenocarcinoma progression, its expression level was also assessed in the present adenocarcinoma series. Notably, *LOX* levels in adenocarcinoma patients were significantly associated with histological subtype (P=0.03), as well as with stage (P<0.0001; Table III) distribution, indicating that *LOX* activation may promote tumor progression. The samples were divided into high and low *LOX* expression groups based on the median *LOX* fold-change value (median fold-change, 132; mean, 141.82±88.9). Survival analysis was performed using the Kaplan-Meier method with the post-operative DFI and OS times as endpoints in order to evaluate the association between *LOX* expression and prognosis in the adenocarcinoma patients. It was identified that adenocarcinoma cases with high *LOX* mRNA expression were associated with significantly shorter median DFI and OS times compared with the cases with low *LOX* expression (P=0.02 and P=0.005, respectively; Fig. 2).

Taken together, these data support *LKB1* signalling as a key pathway in lung adenocarcinoma, with a potential relevant role for *LOX*.

miRNA selection and expression. The microRNA target prediction program (http://www.microrna.org) was used to identify putative miRNA-mRNA interactions in the LKB1 pathway, and subsequently, the impact of the 15 selected miRNAs (miRs -93, -96, -34a, -34c, -214, -33a, -30b, -145, -182, -30c, -183, -29b, -29c, -153 and -138) on the expression of target genes. Fig. 3 depicts the expression profiles across all the adenocarcinoma samples in the two age groups. The younger and older patients shared similar gene expression profiles; any differentially expressed genes in the *LKB1* pathway were associated with modulated miRNA expression, suggesting

Table II. LKB1 expression in the 88 lung adenocarcinoma patients.

Variable	LKB1 expression, mean ± SD	P-value
Age group		0.21
Younger (≤50 years)	173.9±13.4	
Older (>50 years)	149.8±13.4	
Sex		0.03
Male	146.9±11.6	
Female	188.0±15.4	
Prevalent adenocarcinoma pattern		0.24
Lepidic	184.9±16.4	
Solid	137.9±17.3	
Acinar	154.0±18.8	
Papillar	173.1±26.7	
Tumour grading		0.14
G1	244.8±50.9	
G2	166.1±11.5	
G3	143.4±16.9	
Stage		0.01
I	192.2±13.5	
II	129.8±18.2	
III-IV	142.3±16.7	

LKB1, liver kinase B1; SD, standard deviation.

Table III. LOX expression in the 88 lung adenocarcinoma patients.

Variable	LOX expression, mean ± SD	P-value
Age group		0.7
Younger (≤50 years)	137.6±13.4	
Older (>50 years)	146.1±13.4	
Sex		0.05
Male	156.1±11.6	
Female	116.9±15.4	
Prevalent adenocarcinoma pattern		0.03
Lepidic	131.0±16.0	
Solid	162.9±17.0	
Acinar	159.6±18.4	
Papillar	85.0±26.1	
Tumour grading		0.52
G1	147.5±51.5	
G2	134.0±11.7	
G3	157.9±17.1	
Stage		<0.0001
I	102.9±11.9	
II	126.4±16.1	
III-IV	214.8±14.8	

LOX, lysyl oxidase; SD, standard deviation.

that they were the gene targets of the modulated miRNAs. The seed region of miR-93 was predicted to bind to one site, position 287, in the human *LKB1* 3'-UTR (Fig. 4). The samples were divided into high and low miR-93 expression groups based on the median miR-93 fold-change value (median fold-change, 3.933; mean, 4.845.91±3.056); *LKB1* expression was reduced in samples with high miR-93 expression (χ^2 test; P=0.0007; data not shown), indicating that this endogenous miRNA may suppress *LKB1*. No statistically significant association was identified between *CCND1* and miR-93 expression, even though decreased *CCND1* levels tended to be observed in cases with high miR-93 expression (P=0.11) and an alignment at position 1013 of the 3'-UTR was identified (Fig. 4). *LOX* downregulation was observed in the lung adenocarcinoma specimens with high miR-30b expression (P=0.04), and a direct interaction of this miRNA and the 3'-UTR of the *LOX* gene at position 594 was identified (Fig. 4). Other potential miRNA binding sites within the *LOX* 3'-UTR were identified (for miRs -145, -182, -30c, -183, -29b, -29c and -153); potential miRNA binding sites were also identified in *YAP1* (miR-138) and *survivin* (miR-214-3p), but neither of them were indicated to influence the expression levels of their mRNA targets (data not shown).

KRAS mutation analysis. Pyrosequencing analysis was performed to identify mutational hot-spots of the KRAS gene. Among the 88 tumor specimens, 34 tumors (38%) were
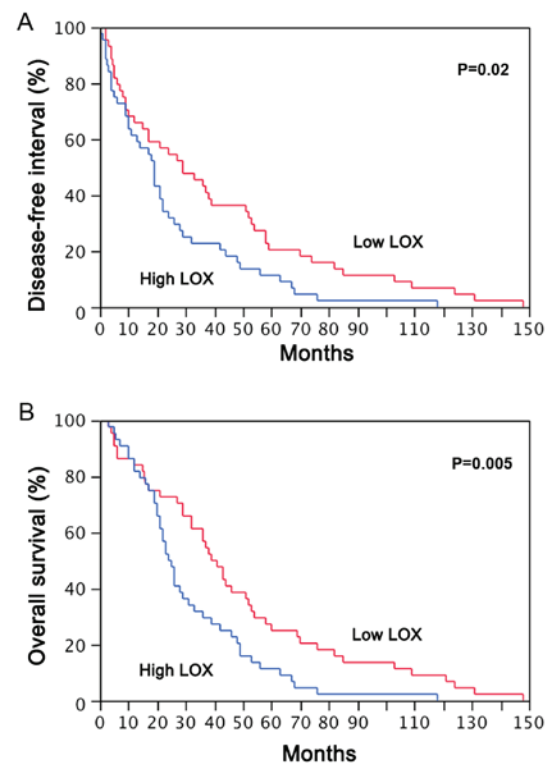

Figure 2. Kaplan-Meier curves for the survival analysis of (A) disease-free interval and (B) overall survival in the 88 adenocarcinoma patients according to *LOX* expression level. LOX, lysyl oxidase.
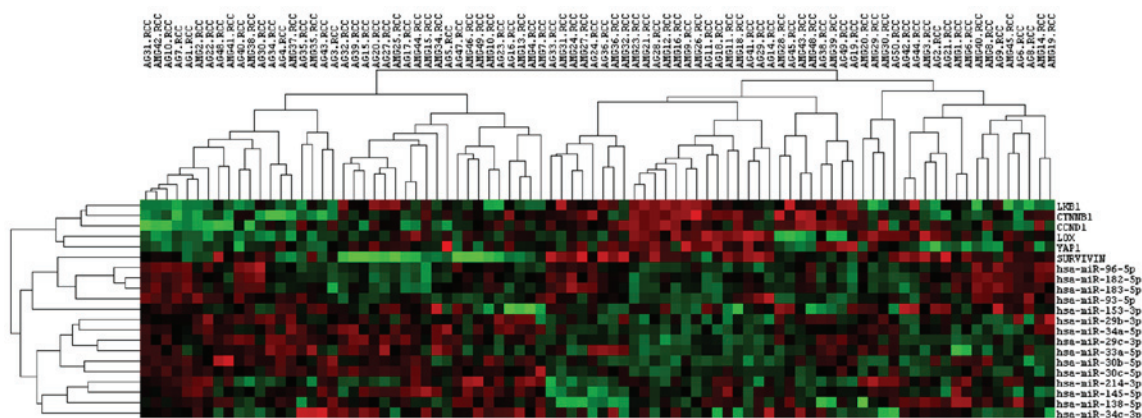
Figure 3. Comparison of gene expression profiles of younger and older lung adenocarcinoma patients. A heat map is presented of unsupervised hierarchical clustering of 6 genes and 15 miRNAs from the Nanostring gene panel based on samples from young (AG) and old (AMG) lung adenocarcinoma patients. Green indicates relatively underexpressed genes; and red indicates relatively overexpressed genes. The genes and miRNAs tested did not separate younger and older patients. miRNA/miR, microRNA; hsa, *homo sapiens*; *LKB1*, LKB1, liver kinase B1; *CTNNB1*, catenin β-1; *CCND1*, cyclin D1; *LOX*, lysyl oxidase; *YAP1*, yes-associated protein 1.
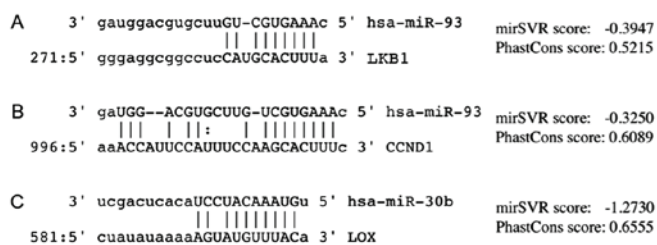

Figure 4. MicroRNA alignment with (A) *LKB1*, (B) *CCND1* and (C) *LOX* mRNA by the target prediction program (http://www.microrna.org). SVR indicates the miRNA-target prediction algorithm based on a supervised vector regression (SVR) model. miR, microRNA; hsa, *homo sapiens*; *LKB1*, liver kinase B1; *CCND1*, cyclin D1; *LOX*, lysyl oxidase.

determined to have point mutations, 13/44 among younger and 21/44 among older patients. However, no significant difference was identified in KRAS mutation distribution between the two age groups (P=0.07; Table I). Concerning the KRAS mutation type, the G12C substitution was present in 15 samples (7 younger and 8 older patients); the G12V point mutation in 11 samples (3 younger and 8 older patients); the G12D mutation in 3 samples (1 younger and 2 older patients); the G12A mutation in 2 samples from the older cohort; the G12S and G13D in 1 sample each among younger patients, and Q61L mutations in only 1 older patient (Table IV).

Discussion

Lung cancer remains the main cause of cancer-related mortality worldwide, and the age at diagnosis has been decreasing in recent years (2,4). Younger patients with lung cancer appear to exhibit distinct clinicopathological features: They are more commonly non-smokers and female, and present a prevalence for adenocarcinoma and advanced disease; however, there is controversy regarding the outcome as it has been reported as improved by certain studies (4,31) and unaffected by others (31-34). Data on the management of adenocarcinoma in the elderly are insufficient (35-39), and thus, whether young lung cancer patients have specific molecular and pathologic

features or different survival outcomes remain unclear. In the present study, 44 lung adenocarcinoma patients ≤50 years old were selected as the younger group and 44 cases >50 years as the older group; a predominance of females was identified in the younger group, and the acinar pattern was most prevalent, which was in accordance with previous studies (4,40-42). There were no significant differences in survival, in terms of DFI and OS, in young lung adenocarcinoma patients compared with the older age group. At present, there is no general consensus on the influence of age on survival, and this issue is open to question. Any discrepancies between reports may be due to the limited number of studies and to the specific cut-off age used to separate younger from older patients; the current study used 50 years old as the cut-off value, according to several previous reports (2,43,44).

An aim of this retrospective study was also to focus on the expression pattern of *LKB1* and its downstream signalling pathways, in order to evaluate their associations with clinicopathological features and prognoses in lung adenocarcinoma, comparing younger patients with their elderly counterparts.

Although there are currently no drugs in routine clinical use that specifically target LKB1, there is a growing number of approaches that may differentially benefit patients with a dysregulated LKB1 pathway (45-49). A critical role for LKB1 has been suggested in catenin-beta1 signalling in lung cancer through its modulation of *CCND1* and *survivin* gene expression (50). Additionally, *LOX* has been reported to be efficiently suppressed by *LKB1*, and *YAP1* has been reported to be initially activated by *LKB1* loss in lung ADC.

A mRNA panel was customized of the 6 abovementioned genes and 15 miRNAs involved in the LKB1 pathway, and expression profiling was performed with NanoString technology, a recently developed platform that can make direct multiplexed measurements of expression through digital readouts of the abundance of mRNA/miRNA transcripts (51). Several studies have indicated that the NanoString technique is a reliable and flexible method for the assessment of gene expression in limited FFPE tissues, and it has exhibited similar results using fresh-frozen tissue (52-54). This field appears to be of importance, since FFPE represents most of the specimens

Table IV. KRAS mutation distribution in the two age groups of lung adenocarcinoma patients.

	Cases, n		
KRAS status	Younger (n=44)	Older (n=44)	Total (n=88)
Wild-type	31	23	54
Mutated	13	21	34
G12C	7	8	15
G12V	3	8	11
G12D	1	2	3
G12A	0	2	2
G12S	1	0	1
G13D	1	0	1
Q61L	0	1	1

collected in routine diagnostic pathology, and it will allow for the connection of clinical follow-up data to large numbers of patients (55); therefore, the NanoString methodology may be readily adapted clinically as a highly reproducible alternative to quantitative polymerase chain reaction and sequencing methods (56).

LKB1 has different molecular targets, and thus, screening of the *LKB1* signalling pathway appears a necessary step to explain its suppressive function in cancer cell biology. No differences in *LKB1* levels and *KRAS* mutation rates were identified between young and older patients, possibly due to the biological heterogeneity of KRAS-mutant lung adenocarcinomas, to the relatively small group size, and/or to other underlying molecular differences such as epidermal growth factor mutations or anaplastic lymphoma kinase translocations (18). Notably, low LKB1 expression was apparent in the solid histological subtype, and high expression in females and early clinical stage, which suggest an important role for LKB1 in inhibiting the growth of lung cancer cells, considering that the solid subtype, male gender and advanced stages are reportedly survival disadvantages in lung adenocarcinoma (57). However, it remains unclear how *LKB1* loss contributes to lung carcinogenesis, and the post-transcriptional regulation of *LKB1* may play a central role. The current results also predicted that miR-93 may be able to downregulate *LKB1* and *CCND1*, consequently leading to the loss of *LKB1*-dependent tumor suppression; such is in agreement with previous studies reporting that high levels of miR-93 (58) and low levels of *LKB1* (59) as well as *CCND1* (60) were correlated with poor survival among lung cancer patients.

The current study also attempted to elucidate the involvement of *LOX*. It is reported that the tumor microenvironment serves a critical role in tumorigenesis (61), and that *LOX*, due to its influence on the cellular microenvironment, may be a target for cancer therapy (62,63). The current data further indicated that aberrant *LOX* expression was involved in lung carcinogenesis and cancer progression, revealing that *LOX* levels in adenocarcinoma patients were significantly associated with overall stage distribution and poor prognosis regardless of

age at diagnosis. Furthermore, the findings indicated that the observed positive prognostic effect of *LOX* was associated, at least in part, to miR-30b regulation, confirming the conclusions by Zhong *et al* (64) regarding a central role of this miRNA in NSCLC suppression. However, the impact of *LOX* remains incompletely clear; it is possible that there exists multiple forms of *LOX* proteins (65), and it is not known whether the signalling components downstream of *LKB1*, including *LOX*, may be involved in an *LKB1*-independent manner; further studies will be required to reach a conclusive point.

The finding that *LKB1* and *LOX* may be repressed by specific miRNAs establishes a regulatory link within the LKB1 tumor suppressor pathway; miRNA-dependent post-transcriptional regulation of *LKB1* may be an alternative to inactivating *LKB1* mutations, which are rare among sporadic tumors (10). Additional study on *LKB1* pathways and other components of the *LKB1* complex may expand knowledge regarding tumor metabolism and growth potential in lung adenocarcinoma.

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Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

LB, MG and GF conceived and designed the experiments. MG performed the experiments. LB wrote the manuscript. GF was responsible for lung cancer diagnosis and contributed to conception and design of the study. FM and ML performed lung surgery and follow-up and contributed to conception and design of the study.

Ethics approval and consent to participate

The current study was conducted in accordance with the ethical standards of Institutional Research Committee of the University of Pisa (Pisa, Italy) and with the 1964 Helsinki declaration; informed consent for the tissue collection and molecular analysis was collected by the oncologist upon each patient's first visit from January 2003 to December 2013 to the University Hospital of Pisa (Pisa, Italy).

Patient consent for publication

Informed consent collected from all patients permitted the use of their tissues for research purposes.

Competing interests

The authors declare that they have no competing interests.

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