British Journal of Cancer (2008) 99, 245–252

www.bjcancer.com

Mutations in the LKB1 tumour suppressor are frequently detected in tumours from Caucasian but not Asian lung cancer patients

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Somatic mutations of *LKB1* tumour suppressor gene have been detected in human cancers including non-small cell lung cancer (NSCLC). The relationship between *LKB1* mutations and clinicopathological characteristics and other common oncogene mutations in NSCLC is inadequately described. In this study we evaluated tumour specimens from 310 patients with NSCLC including those with adenocarcinoma, adenosquamous carcinoma, and squamous cell carcinoma histologies. Tumours were obtained from patients of US ($n=143$) and Korean ($n=167$) origin and screened for *LKB1*, *KRAS*, *BRAF*, and *EGFR* mutations using RT—PCR-based SURVEYOR-WAVE method followed by Sanger sequencing. We detected mutations in the *LKB1* gene in 34 tumours (11%). *LKB1* mutation frequency was higher in NSCLC tumours of US origin (17%) compared with 5% in NSCLCs of Korean origin ($P=0.001$). They tended to occur more commonly in adenocarcinomas (13%) than in squamous cell carcinomas (5%) ($P=0.066$). *LKB1* mutations associated with smoking history ($P=0.007$) and *KRAS* mutations ($P=0.042$) were almost mutually exclusive with *EGFR* mutations ($P=0.002$). The outcome of stages I and II NSCLC patients treated with surgery alone did not significantly differ based on *LKB1* mutation status. Our study provides clinical and molecular characteristics of NSCLC, which harbour *LKB1* mutations.
British Journal of Cancer (2008) **99,** 245–252. doi:10.1038/sj.bjc.6604469 www.bjcancer.com
Published online 1 July 2008

Keywords: carcinoma; non-small cell lung; mutation; LKB1; EGFR; KRAS

Peutz–Jeghers syndrome (PJS) is caused by mutations in the *LKB1* tumour suppressor gene (Hemminki *et al*, 1998). LKB1 is serine–threonine kinase, which has been shown to regulate cell cycle progression, apoptosis, and cell polarity (Tiainen *et al*, 1999). The major target of LKB1 kinase activity is thought to be AMP-activated protein kinase (AMPK). AMPK is activated under low cellular energy conditions by raising AMP levels and it phosphorylates multiple downstream targets including tuberosclerosis complex 2 gene, which represses mTOR signalling. Phosphorylation of AMPK by LKB1 is needed for full activity of AMPK and suppression of mTOR activity under low energy conditions (Shaw *et al*, 2004). The hallmarks of PJS include mucocutaneous pigmentation and hamartomatous polyps of the gastrointestinal tract. Patients with PJS have an increased risk of developing gastrointestinal, pancreatic, breast, gynecological, and non-small cell lung cancers (NSCLC). The overall risk for cancers is increased 5- to 12-fold in different age groups compared with the general population (Hearle *et al*, 2006). Somatic mutations of the *LKB1* tumour suppressor have rarely been found in cancers from patients who do not have PJS except for NSCLC (Avizienyte *et al*, 1999). Previous reports have suggested the *LKB1* mutation rate to be as high as 30% in NSCLC tumours and cell lines derived from patients of Caucasian origin (Carretero *et al*, 2004; Matsumoto *et al*, 2007) and to be infrequent in NSCLC patients of Asian origin (3%) (Onozato *et al*, 2007). Furthermore, *LKB1* mutations have been shown to be associated with adenocarcinoma histology, male gender, and smoking history (Matsumoto *et al*, 2007). A recent report of using a mouse model for *lkb1* inactivation in NSCLC has provided insights into the role of the gene in this cancer. This study showed that *lkb1* inactivation in combination with activating mutations of *kras* using inducible promoters in the lung was associated with decreased survival compared with *kras* mutation alone (Ji *et al*, 2007).

Current screening techniques for *LKB1* tumour suppressor mutations rely on conventional exonic sequencing of the DNA, which can identify single base pair changes and small deletions/insertions (Ballhausen and Gunther, 2003). The addition of multiple ligation-dependent probe amplification (MLPA), which enables detection of exonic and whole gene deletions, with exonic sequencing has increased the mutation detection rates to 80% in patients with PJS phenotype (Volikos *et al*, 2006). Conventional

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Revised 9 May 2008; accepted 28 May 2008; published online 1 July 2008

sequencing has also been used to detect mutations of *LKB1* at mRNA level and some mutations missed by sequencing at the DNA level have been discovered by mRNA-based approaches (Abed *et al*, 2001). However, mutant forms of *LKB1* mRNAs can have a shortened half-life because of nonsense-mediated decay, which can potentially interfere with mutation detection (Abed *et al*, 2001).

We have recently described a rapid and sensitive enzymatic method to detect mutations in epidermal growth factor (*EGFR*) of DNA from fresh tissue and paraffin-embedded tissues (Janne *et al*, 2006). This method includes amplification of region of interest with PCR, SURVEYOR endonuclease digestion of the products, which cleaves mismatched heteroduplex DNAs, and detection of DNA fragments by sensitive high-performance liquid chromatography (HPLC) WAVE HS system. Subsequently, SURVEYOR-positive specimens are fractionated in partially denaturing conditions and are Sanger-sequenced. The major advantages of SURVEYOR-WAVE method are the fast exclusion of wild-type specimens without laborious conventional sequencing and high sensitivity. The SURVEYOR-WAVE method is more sensitive than conventional sequencing as it can detect mutant DNA sequences when they are present in 1% or more of total DNA (Janne *et al*, 2006).

The current study was designed to analyse the incidence of *LKB1* mutations in NSCLC. Furthermore, we wanted to investigate the *LKB1* mutational frequency in different histologies and ethnic backgrounds, and assess their correlation to smoking history, gender, stage, survival, and other oncogenic mutations in NSCLC.

MATERIALS AND METHODS

Cell lines and tumour specimens

The NSCLC cell lines A549, NCI-H1395, NCI-H1650, NCI-H1666, NCI-H1781, NCI-1975, NCI-H23, NCI-H2126, NCI-H441, NCI-H820, HCC2935, HCC4006, and HCC827 were purchased from ATCC (Manassas, VA, USA). H3255, H3255GR, HCC2279, and PC-9 have been previously described (Ono *et al*, 2004; Tracy *et al*, 2004; Engelman *et al*, 2006). Ma1, and Ma70 are NSCLC cell lines harbouring *EGFR* mutations that were established at the Kinki University, Osaka, Japan. A549, NCI-H1395, NCI-H1666, NCI-H23, NCI-H2122, NCI-H2126, and NCI-H460 have previously been reported to contain *LKB1* mutations (Sanchez-Cespedes *et al*, 2002; Bamford *et al*, 2004; Carretero *et al*, 2004).

NSCLC tumours ($n=310$) were collected from surgical resections from patients with stages I–IV NSCLC when sufficient material for RNA extraction was available. The majority of the specimens ($n=167$) was collected at the Samsung Medical Center, Seoul, Korea. Frozen tumour tissues were collected from 809 out of 2442 patients who underwent curative resection for NSCLC from November 1995 to February 2007 at Samsung Medical Center. One or two pieces from the periphery of the tumour masses – avoiding necrotic regions – were immediately frozen at −80°C until retrieved. Medical records and haematoxylin and eosin-stained slides of the specimen were reviewed by a single pathologist. Only frozen tumour tissues from adenocarcinoma or squamous cell carcinoma (according to the 2004 World Health Organization histopathological criteria) were included. Only frozen tumour tissues with a tumour cell content of more than 70% were used for further analysis. In addition, frozen tumour tissues of the following patients were excluded from the study: patients who had received preoperative neoadjuvant treatments, patients with double primary lung cancer, and patients who had undergone incomplete resections or who had not been subjected to mediastinal lymph node dissections. Selected frozen tumour tissues were used for the microdissection. Briefly, frozen tissues were lightly stained with haematoxylin–eosin to improve visualisation, and necrotic tumour tissues and intervening normal tissues were removed. Each of the microdissected tumour tissues with a tumour cell content of more than 90% was placed in 1 ml Easy Blue reagent of a commercially available RNA isolation kit (easy-spin™ Total RNA Extraction Kit, iNtRON Biotechnology, Gyeonggi-do, Korea), immediately homogenised by vortexing, and the total RNA was extracted. The quantity and quality of RNA were analysed using a spectrometer (Nanodrop Technologies, Rockland, DE, USA) and Agilent 2100 Bioanalyzer (Agilent RNA 6000 Nano Kit, Agilent Technologies Inc., Böblingen, Germany), respectively. Finally, 167 frozen tissues with acceptable quality of RNA (RNA Integrity Number (RIN) value over 7.0) were used for the current studies. All patients provided written informed consent.

The tumours from Caucasian patients ($n=143$) were collected at the Brigham and Women's Hospital, Boston, MA, USA between 1991 and 1997 and have been previously published for patient characteristics and histology, and for expression profile-based clustering of the tumours (Bhattacharjee *et al*, 2001; Hayes *et al*, 2006). Frozen samples of resected lung tumours were obtained within 30 min of resection and subdivided into 100 mg samples and snap frozen at −80°C. Each specimen was associated with an immediately adjacent sample embedded for histology in an optimal cutting temperature medium and stored at −80°C. Six micrometres of frozen sections of embedded samples stained with haematoxylin and eosin were used to confirm the postoperative pathological diagnosis and to estimate the cellular composition of adjacent samples. All specimens underwent pathological review by two pathologists. In all 109 tumours obtained during the same time period were excluded because they did not meet one or more of the eligibility criteria. Tissue samples were homogenised in Trizol (Life Technologies, Gaithersburg, MD, USA) and RNA was extracted and purified by using the RNeasy column purification kit (Qiagen, Chatsworth, CA, USA). Denaturing formaldehyde gel electrophoresis followed by northern blotting using a β-actin probe assessed RNA integrity. Samples were excluded if β-actin was not full length. All patients provided written informed consent. The US cohort included specimens that have previously undergone analyses and the results have been published for *EGFR*, *KRAS*, and *BRAF* mutations (Bhattacharjee *et al*, 2001; Naoki *et al*, 2002; Hayes *et al*, 2006). We reconfirmed the mutations in 30 of these specimens using the SURVEYOR-based analysis (see section SURVEYOR digestion and HPLC analysis) and found 100% concordance between the two methods (data not shown).

Cell line specimens were snap frozen and stored at −80°C. RNA was extracted from tumours and cell lines using Trizol (Invitrogen, Carlsbad, CA, USA), purified with Rneasy Mini Kit (Qiagen, Valencia, CA, USA) and was used for cDNA synthesis using the QuantiTect reverse transcription kit (Qiagen, Valencia, CA, USA).

PCR primers and cycling conditions

For *LKB1* gene analysis, PCR primers were designed to amplify the cDNA in two amplicons. PCR primers of the first amplicon were designed to hybridise to the noncoding area of the mRNA upstream of exon 1 (5′-agggaagtcggaacacaagg-3′) and to exon 5 (5′-ccagatgtccaccttgaagc-3′) generating a PCR product of 797 bp. The primers for the second amplicon located at exon 5 (5′-aacggcc tggacaccttct-3′) and to noncoding exon 10 (5′-gaaccggcaggaagact gag-3′) generating a product of 702 bp, which has an overlapping part with first amplicon. For SURVEYOR-WAVE analysis of *KRAS*, PCR primers (5′-ggcctgctgaaaatgactga-3′, 5′-tcctgagcctgttttgtgtct-3′) were designed to generate an amplicon of 407 bp covering codons 12, 13 and 61, which are the codons commonly mutated in lung cancers. For SURVEYOR-WAVE mutation analysis of *BRAF*, cDNA was amplified in two overlapping amplicons (5′-aggattt cgtggtgatggag-3′, 5′-gatgacttctggtgccatcc-3′, and 5′-gacgggactcgagt gatgat-3′, 5′-ggtatcctcgtcccaccata-3′) covering codons 387–673. For SURVEYOR-WAVE analysis of *EGFR*, PCR amplification was done in a single amplicon (5′-ggagcctcttacacccagtg-3′,

5′-aggtcatcaactcccaaacg-3′), which covered exons 18–21 of the gene. PCR amplification was done using JumpStart Taq (Sigma, St Louis, MO, USA) under the manufacturer's guidelines. A part of the specimens ($n = 103$) was previously characterised for *KRAS*, *BRAF*, *EGFR* mutations using reverse transcritase (RT)–PCR and direct sequencing of the PCR products (Naoki *et al*, 2002; Hayes *et al*, 2006).

SURVEYOR digestion and HPLC analysis

SURVEYOR digestion and HPLC analysis were carried out as described previously (Janne *et al*, 2006). In brief, PCR products were digested in reaction mixture containing equal volumes of SURVEYOR enzyme (Transgenomics, Omaha, NE, USA) and Enhancer (Transgenomics, Omaha, NE, USA) at 42°C for 20 min followed by termination of the reaction by Stop Solution (Transgenomics, Omaha, NE, USA). Specimens were then loaded to the WAVE HS HPLC (Transgenomics, Omaha, NE, USA) at 50°C, eluted with an increasing acetonitrile gradient, and detected by UV detector using DNA intercalating fluorescence dye (Transgenomics, Omaha, NE, USA). When cell lines known to be homozygous for specific mutation were analysed, PCR products were mixed 1:1 with PCR products of a wild-type cell line, denatured by heating, and slowly renatured to generate heteroduplexes.

Sequencing and fractionation

Specimens that showed an altered pattern on the SURVEYOR tracings were purified using QIAquick kit (Qiagen, Valencia, CA, USA) and sequenced bi-directionally by molecular biology core facility of Dana–Farber Cancer Institute. If a specimen showed an altered pattern on the SURVEYOR tracing but had a wild-type sequence by direct DNA sequencing, it underwent fractionation by WAVE HS HPLC in partially denaturing conditions. Running temperatures for specific amplicons were calculated by the Navigator Software (Transgenomics, Omaha, NE, USA). Collected fragments were amplified with PCR using the same primers as in the original amplification, purified and sequenced as previously described above.

Statistical analysis

Fisher's exact test was used to assess the association of *LKB1* mutation status with other clinical, pathological, and genetic characteristics. To adjust for any difference between ethnic groups, the association between *LKB1* mutation rate and each characteristic was also evaluated as stratified contingency tables. If we did not reject that the odds ratios were the same across ethnic groups, we then tested whether the common odds ratios were unity based on the stratified Mantel–Haenszel estimate (Breslow and Day, 1980). Overall survival was estimated using the Kaplan–Meier method, with differences between the groups compared using the log-rank test. All *P*-values were based on a two-sided hypothesis, with $P < 0.05$ considered to be statistically significant and $0.05 < P < 0.10$ considered to be borderline significant.

RESULTS

SURVEYOR-WAVE mutation detection of *LKB1* tumour suppressor in NSCLC cell lines

The impact of the stability of *LKB1* mRNA on detecting *LKB1* mutations was tested using RT–PCR with mRNA extracted from NSCLC cell lines that had previously been characterised for *LKB1* mutations. These included NCI-H441 (wild type) and A549, NCI-H1395, NCI-H23, and NCI-H2126 (all containing *LKB1* mutations). Reverse transcriptase–PCR amplification of the whole coding region of the *LKB1* mRNA showed that cell lines with nonsense (A549, NCI-H23) mutations or 1 bp deletion (H1395) expressed mRNA with comparable size to the wild-type H441 cell line (1460 bp). H2126 cell line, which is known to have homozygous deletion of exons 4–6, expressed mRNA with substantially smaller size (~1000 bp) corresponding to deletion of 398 bp. RT–PCR revealed no major difference in *LKB1* mRNA expression levels between *LKB1* mutant or wild-type cell lines (Figure 1A).

As *LKB1* mutant and wild-type cell lines expressed comparable amounts of *LKB1* mRNA with RT–PCR, we studied the cDNA for mutations using the SURVEYOR-WAVE method. The WAVE HPLC provides a system to analyse DNA fragments smaller than 900 bp and therefore we designed two overlapping amplicons covering exons 1–5 (797 bp) and 5–9 (702 bp) to amplify the whole coding region of *LKB1* mRNA. PCR products of *LKB1* mutant cell lines were mixed 1:1 with the products from wild-type cell lines (H441) to generate heteroduplexes as *LKB1* mutant cell lines were previously reported to be homozygous for the inactivation of the gene. SURVEYOR-WAVE analysis of the amplicon covering exons 1–5 revealed novel peaks with the cDNA for A549, and NCI-H1395 cell lines compared with the wild type from NCI-H441 (Figure 1B). SURVEYOR-WAVE analysis of exons 5–9 showed novel peaks for the NCI-H23 cell line as well. The mutations detected with SURVEOR-WAVE were confirmed by conventional DNA sequencing and they corresponded to previous reports (Sanchez-Cespedes *et al*, 2002; Carretero *et al*, 2004). We could not detect the *LKB1* mutation of H2126 cell line with SURVEYOR-WAVE method using a two-amplicon approach because this cell line has a homozygous deletion of exons 4–6 and the reverse primer of the first amplicon and the forward primer of the second amplicon, which lie on the deleted part of the gene (data not shown).

LKB1 tumour suppressor gene mutations in NSCLC tumours

We next used the SURVEYOR-WAVE method to screen NSCLC tumour specimens ($n = 310$) for *LKB1* mutations. We detected 34 *LKB1* mutations (11%) in the NSCLC tumour specimens (Table 1). The majority of the *LKB1* mutations detected was deletions or insertions ($n = 25$, 74%). The remainder was missense ($n = 7$, 21%) and nonsense ($n = 2$, 6%) mutations (Table 2, Figure 1C). About one-half of the deletions and insertions were small, covering <15 bp ($n = 14$, 56%), whereas larger deletions ($n = 11$, 44%) covering hundreds of base pairs were detected in the remaining specimens. Some mutational hotspots were discovered. The areas that had the same mutation in more than one tumour specimen included deletion of exon 4 ($n = 4$), deletion of exons 2 and 3 ($n = 3$), D194Y ($n = 2$), and P281L ($n = 2$). Interestingly, a significant portion of the mutations was located in exon 1 ($n = 11$, 32%) but there was no area of recurrent mutations in this exon (Table 2). Of the missense mutations detected in the current study, all except R426W are in the kinase domain of the protein. Missense mutations in codons 176 and 194 have been previously characterised in PJS (Launonen, 2005). We also found four F354L alterations (data not shown) but we did not consider these as missense mutations as this alteration has previously been reported to be a rare polymorphism of the gene (Launonen *et al*, 2000). We did not have access to the corresponding normal tissues and therefore, we could not verify if some of the missense mutations were somatic or germline.

Association of *LKB1* tumour suppressor mutations in NSCLC with clinicopathological characteristics

The mutation frequency of *LKB1* gene was significantly higher in NSCLCs in the Caucasian cohort (Table 1). Twenty-five (17% of specimens) of the *LKB1* mutations were detected in NSCLCs

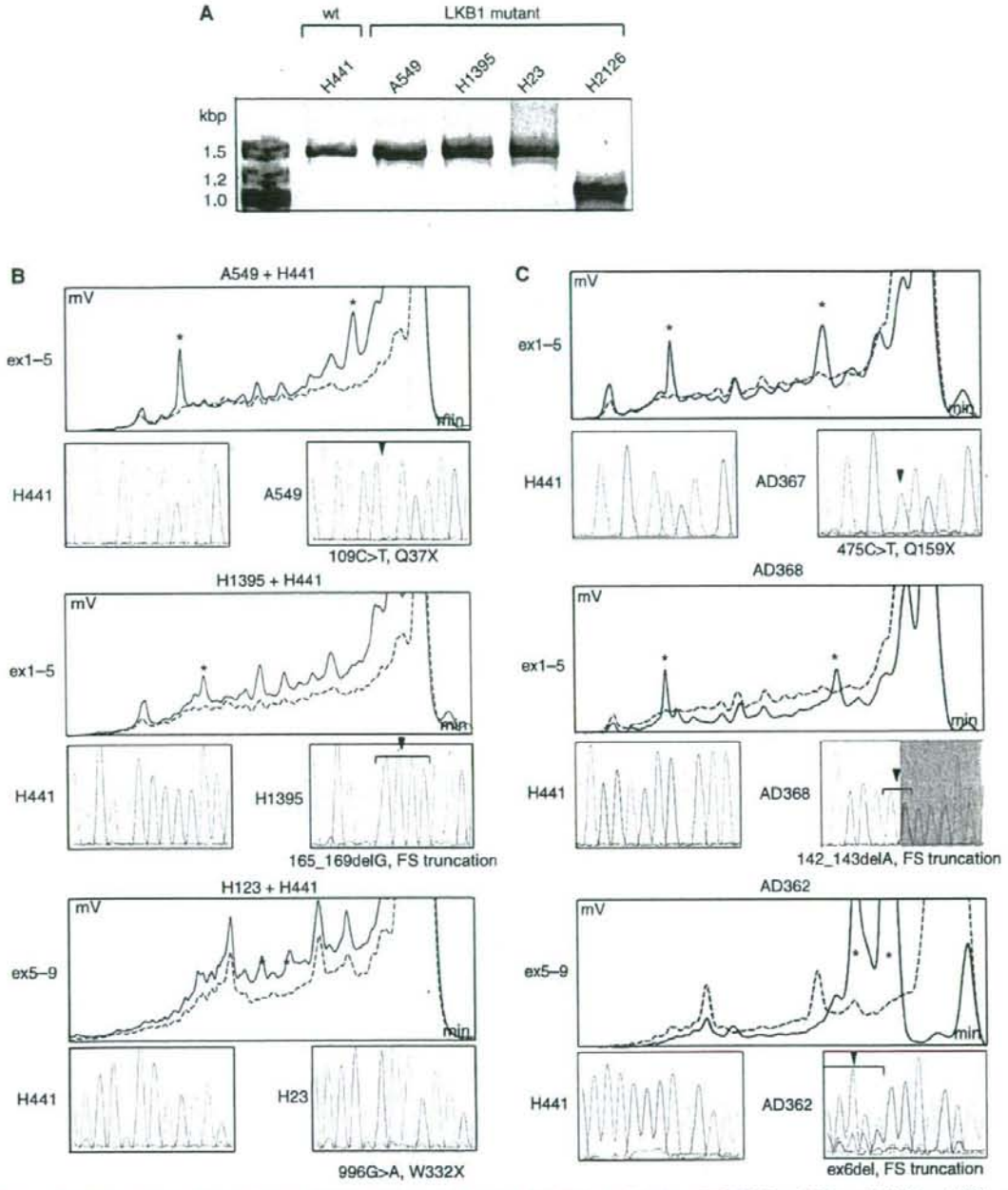

Figure 1 Mutation analysis of *LKB1* gene in NSCLC cell lines and tumours. RT–PCR amplification of cDNA from *LKB1* wt (H441) and *LKB1* mutant (A549, H1395, and H23) cell lines display the full length LKB1 mRNA (1.4 kbp) while the *LKB1* mutant cell line, H2126 with a deletion of exons 4–6 expresses a shorter mRNA (1.0 kbp) (**A**). HPLC tracings of SURVEYOR-WAVE mutation analysis of NSCLC cell lines A549, H1395, or H23 (continuous line), and H441 (dashed line). Time in minutes is shown on the *X*-axis, voltage in mV on the *Y*-axis (**B**). A549 and H1395 show novel peaks (*) in the amplicon covering exons 1–5 (ex1–5) corresponding to 109C>T, Q37X and 165_169delG, frameshift and truncation (FS truncation) mutations. The analysis from H23 demonstrates novel peaks in the amplicon covering exons 5–9 (ex5–9) corresponding to 996G>T, W332X mutation. *LKB1* wild-type cDNA (H441) was added to PCR products 1 : 1, denatured by heating and slowly renaturated to generate heteroduplexes since A549, H1395, and H23 have previously reported to be homozygous for the *LKB1* mutations. SURVEYOR-WAVE mutation analyses of NSCLC tumours (**C**). AD367 and AD368 tumours showed novel peaks in the ex1–5 amplicon corresponding to 475C>T, Q159X, and 142_143delA, FS, truncation mutations. AD362 tumour had novel peaks in ex5–9 amplicon corresponding to deletion of exon 6. Mutant sequences for AD367 and AD368 are displayed from sequences using the forward primer while mutation of the AD362 is showed with reverse primer.

Table 1 Frequency of *LKB1* mutations in NSCLC tumours and their association with clinicopathological characteristics

	LKB1 mutation		
	+	–	P-value*
All tumours	34 (11%)	276 (89%)	
Age, median	61.2	62.2	
Ethnicity			
Caucasian cohort	25 (17%)	118 (83%)	0.001
Asian cohort	9 (5%)	158 (95%)	
Gender			
Male	20 (11%)	167 (89%)	NS
Female	14 (12%)	107 (88%)	
Smoking			
Never (<10 py)	2 (3%)	70 (97%)	0.007
Smoker (>10 py)	26 (14%)	161 (86%)	
Tumour stage			
I	19 (10%)	169 (90%)	NS
II	8 (14%)	51 (86%)	
III	5 (11%)	42 (89%)	
IV	1 (12%)	7 (88%)	
Histology			
Adenocarcinoma	27 (13%)	180 (87%)	0.047
Squamous carcinoma	5 (5%)	87 (95%)	
Adenosquamous	2 (22%)	7 (78%)	

*Fisher's exact test. NS = not statistically significant ($P > 0.05$).

collected from patients in the United States, whereas only nine mutations (5% of specimens) were detected in the Korean cohort ($P = 0.001$) (Table 1). The *LKB1* mutation rate tended to be higher in adenocarcinomas (13%) compared with squamous cell carcinomas (5%) ($P = 0.067$). Differences in histological subgroups were relatively modest in the US cohort with mutations in 18 out of 94 (19%) adenocarcinomas *vs* 5 out of 38 (13%) in squamous cell cancers ($P = 0.461$). This is in contrast to the findings in the Asian patients where all of the *LKB1* mutations were detected in adenocarcinomas (9 out of 113 (8%)) and none were detected in squamous cell cancers (0 out of 54 (0%); $P = 0.032$). Nevertheless, the higher rate of *LKB1* mutation in adenocarcinomas compared with squamous cell carcinomas retains the same level of statistical significance (stratified $P = 0.064$) after adjusting for fluctuation between ethnic groups. The US cohort also included nine specimens from adenosquamous carcinomas and two out of nine (22%) had *LKB1* mutations, which is similar to the frequency in adenocarcinomas in this population (Table 1). There was no association between *LKB1* mutations and the clinical stage of the NSCLC patients. Kaplan–Meier survival curves of stages I and II NSCLC patients showed a tendency for shorter survival in patients with *LKB1* mutant tumours but this, however, did not reach statistical significance ($P = 0.17$) (Figure 2). No differences in survival were observed in patients who harboured both *LKB1* and *KRAS* mutations compared with those with *KRAS* or *LKB1* alone but the total number of patients with both mutations who had stages I or II NSCLC was small ($n = 9$; data not shown). We detected an association of *LKB1* mutations with a smoking history ($P = 0.007$) and only two mutations were detected in tumours from 72 NSCLC patients who were either never or light (≤10 pack years) former smokers (Table 1). After adjusting for ethnic group, the higher rate of *LKB1* mutation among patients with a smoking history is borderline significant (stratified $P = 0.067$). The reduction in statistical significance is likely owing to the loss of power associated with the overall rarity of *LKB1* mutations among never or light former smokers. For these analyses we combined both never smokers and light (≤10 pack years) smokers as the frequency of mutations in other oncogenes such as *EGFR* is similar in these two patient groups (Pham *et al*, 2006). There were no correlations between *LKB1* mutations and gender or age of a patient.

Table 2 The specific *LKB1* mutations in NSCLC tumours

Mutation type	No. (%)	Mutation	Amino acid change	Exon	Histology
Missense	7 (21)	*526G>T	D176Y	4	Ad
		*580G>T	D194Y	4	Ad
		580G>T	D194Y	4	Sq
		829G>T	D277Y	6	AdSq
		*842C>T	P281L	6	Ad
		*842C>T	P281L	6	Ad
		1276C>T	R426W	9	Ad
Nonsense	2 (6)	206C>A	S69X	1	Ad
		475C>T	Q159X	4	Ad
Deletion/ insertion	25 (74)	*75_76del2&insT	FS, truncates	1	Ad
		120_130del11	FS, truncates	1	Ad
		125_127insGG	FS, truncates	1	Ad
		128_129delC	FS, truncates	1	Ad
		142_143delA	FS, truncates	1	Ad
		180delC	FS, truncates	1	Ad
		209delA	FS, truncates	1	Ad
		227_228delC	FS, truncates	1	Ad
		47_651del604	FS, truncates	1-5	Sq
		153_536del384	FS, truncates	1-4	AdSq
		*exon 2-3del	Truncates	2-3	Sq
		*exon 2-3del	Truncates	2-3	Ad
		exon 2-3del	Truncates	2-3	Ad
		exon 2-4del	FS, truncates	2-4	Sq
		464_465del2insTTTGCT	FS, truncates	3-4	Sq
		562_563delG	FS, truncates	4	Ad
		*exon 4del	FS, truncates	4	Ad
		exon 4del	FS, truncates	4	Ad
		exon 4del	FS, truncates	4	Ad
		exon 4del	FS, truncates	4	Ad
		610_623del14	FS, truncates	5	Ad
		*837_844delC	FS, truncates	6	Ad
		837_844insC	FS, truncates	6	Ad
		exon 6del	FS, truncates	6	Ad
		1038_1040insG	FS, truncates	8	Ad

Ad = Adenocarcinoma; AdSq = Adenosquamous carcinoma; Sq = Squamous cell carcinoma; . *These mutations were detected in Korean NSCLC patients.

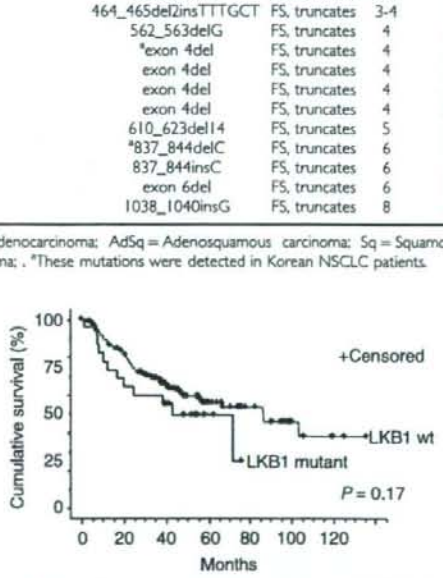

Figure 2 Kaplan–Meyer survival curves of stage I and II NSCLC patients with LKB1 wildtype (red line, $n = 198$) *vs* LKB1 mutant (blue line, $n = 23$) tumours.

Association of *LKB1* mutations with *K-Ras*, *B-Raf*, and *EGFR* mutations in NSCLC

Previous reports have suggested that in NSCLC cell lines, *LKB1* mutations often occur concurrently with *KRAS* or *BRAF* mutations

(Sanchez-Cespedes *et al*, 2002; Carretero *et al*, 2004). Furthermore, *EGFR* mutations are often mutually exclusive with *KRAS* mutations in NSCLC (Kosaka *et al*, 2004; Marchetti *et al*, 2005). We used combined data from previous papers (Sanchez-Cespedes *et al*, 2002; Carretero *et al*, 2004) and from Sanger institute's databases (Bamford *et al*, 2004) to analyse association of *LKB1* mutations with mutations of *KRAS*, *BRAF*, and *EGFR*. Analysis of *LKB1* mutation harbouring NSCLC cell lines (A-427, A549, NCI-H1395, NCI-H1666, NCI-H2122, NCI-H2126, NCI-H23, and NCI-H460) showed that five of the cell lines (63%) had concurrent *LKB1* and *KRAS* mutations, two (25%) had concurrent *LKB1* and *BRAF* mutations, and only one (13%) had neither *KRAS* nor *BRAF* mutations. None of these cell lines had EGFR mutations.

As our findings in NSCLC cell lines suggested concurrency of *KRAS* or *BRAF* and mutual exclusiveness of *EGFR* mutations with LKB1 mutations, we analysed the mutational status of these genes in our primary NSCLC tumour specimens. *KRAS* mutations were detected in 49 (16% in the whole tumour set, 25% in Caucasian and 8% in Asian specimens) tumour specimens with 10 (20% of *KRAS* mutants) of these occurring concurrently with an *LKB1* mutation ($P=0.042$) (Table 3). Four *BRAF* mutations (1%) were found in the tumour set (G465V, N581S, L596R, and T599I) and one of these (N581S) occurred concurrently with *LKB1* mutation ($P=0.373$). Seventy tumours (23% in the whole tumour set, 9% in Caucasian, 34% in Asian specimens) had *EGFR* kinase domain mutations with only one of them occurring concurrently with an *LKB1* mutation ($P=0.002$). The tumour with a concurrent *EGFR* and *LKB1* mutation had a missense mutation of *LKB1* outside the kinase domain (R426W). No germ line DNA was available from this patient. However, a recent report has suggested that R426W is in fact a rare polymorphism of the gene (Onozato *et al*, 2007). Taken together our findings suggest that unlike *KRAS*, mutations in *EGFR* and *LKB1* are mutually exclusive in NSCLC.

Previous reports (Sanchez-Cespedes *et al*, 2002; Carretero *et al*, 2004) and Cancer Genome Project by Sanger Institute (Bamford *et al*, 2004) have extensively characterised *LKB1* mutations in NSCLC cell lines with *KRAS* and *BRAF* mutations, but *LKB1* status of *EGFR* mutant NSCLC cell lines has not been extensively analysed. Therefore, we analysed the *LKB1* genotype of NSCLC cell lines with known *EGFR* or *ERBB2* mutations. Twelve *EGFR* mutant and one *ERBB2* mutant cell lines were analysed for *LKB1* genotype. No *LKB1* mutations were detected in these cell lines (Table 4).

Table 3 Association of *LKB1* mutations with *KRAS*, *BRAF*, and *EGFR* mutations in NSCLC tumours

		LKB1 mutation		
		+	–	*P*-value*
EGFR mutation	+	1	69	0.002
	–	33	207	
K-Ras mutation	+	10	39	0.042
	–	24	237	
B-Raf *mutation*	+	1	3	0.373
	–	33	273	

*Fisher's exact test.

Table 4 LKB1 genotypes of NSCLC cell lines with EGFR or ERBB2 mutations

Cell line	*EGFR* genotype	*HER2* genotype	*LKB1* genotype
H1650	E746_A750del	Wt	Wt
H1781	Wt	G776V, Cins	Wt
H1975	L858R, T790M	Wt	Wt
H3255	L858R	Wt	Wt
H3255GR	L858R, T790M	Wt	Wt
H820	L747_L751del, T790M	Wt	Wt
HCC2279	E746_A750del	Wt	Wt
HCC2935	E746_T751del, S752I	Wt	Wt
HCC4006	L747_E749del, A750P	Wt	Wt
HCC827	E746_A750del	Wt	Wt
Ma-1	E746_A750del	Wt	Wt
Ma-70	L858R	Wt	Wt
PC-9	E746_A750del	Wt	Wt

Wt = wild type.

DISCUSSION

The present study characterised *LKB1* mutation frequency in NSCLC using one of the largest tumour sets to date ($n=310$). Our study analysed tumours from different histologies and of both a US and Korean origin to determine potential histological and ethnic variation in *LKB1* mutational frequency. The large size of our study enabled us to study associations of *LKB1* mutations with clinocopathological factors, which have been incompletely characterised in previous studies (Sanchez-Cespedes *et al*, 2002; Carretero *et al*, 2004; Fernandez *et al*, 2004; Matsumoto *et al*, 2007; Onozato *et al*, 2007). In addition, we used a modification of a sensitive mutation screening technique that we have previously developed to facilitate the rapid detection of *LKB1* mutations (Janne *et al*, 2006).

Findings from our study confirm the high frequency of *LKB1* mutations in NSCLC (11%), which in contrast, are rare (0–4%) in other common solid malignancies (Avizienyte *et al*, 1998, 1999). The reason behind these observations is presently unknown but might reflect the differences in carcinogen exposure in the lungs compared with other tissues. In support of this hypothesis, we find that *LKB1* mutations are significantly ($P=0.007$) more common in smokers than in never or light ($\leqslant$10 pack years) cigarette smokers (Table 1). Male PJS patients (age $\geqslant$50 years) have an increased risk of developing lung cancer compared with the general population but the relationship of smoking and the increased risk of lung cancer in PJS is unknown (Hearle *et al*, 2006). Interestingly, the *LKB1* mutation spectrum found in the current study is very similar to those previously published for PJS (deletions 34%, insertions 15%, splice site mutations 14%, missense mutations 21%, and nonsense mutations 12%) (Launonen, 2005) and, as in PJS, no clear mutational hotspots were detected.

Our study also demonstrated that the *LKB1* mutation frequency was significantly higher in cancers derived from a US population compared with those found in Korean patients (17 *vs* 5%; $P=0.001$). These differences also track with cigarette smoking, as the number of never/light former smokers was much higher in the Korean cohort compared with the US cohort of patients (38 *vs* 13%). Similarly, a recent study of 100 Japanese NSCLCs found that only 3% contained an *LKB1* mutation (Onozato *et al*, 2007). These findings are in contrast to *EGFR* mutations, which are more frequently detected in tumours from never/light cigarette smokers and from Asian patients (Janne and Johnson, 2006). Our studies further highlight ethnic and environmental differences in the origins of NSCLC.

Given the differences in *LKB1* mutation frequencies in smokers *vs* never/light smokers and in the US compared with Korean patients, we further determined whether these were also associated with other oncogene mutations known to vary in these subgroups of patients. Consistent with prior studies we found a significant association with concurrent *KRAS* mutations, which are common in smokers (Ahrendt *et al*, 2001), in one out of three of NSCLC with *LKB1* mutations (Table 3). In contrast, there was a significant inverse relationship of *LKB1* mutations with *EGFR* mutations in both NSCLC tumours and cell lines, which has not previously been

described (Tables 3 and 4). These differences may relate to the biological role of LKB1 in lung cancer. It is possible that in *EGFR* mutant lung cancers there is already maximal activation of the PI3K/Akt/mTOR signalling pathway and thus an *LKB1* mutation may not be required to further potentiate this signalling pathway. In contrast in *KRAS* mutant cancers, a concurrent *LKB1* mutation may be required to enhance mTOR activation. Mice with concurrent *KRAS* mutations and *LKB1* inactivation have more aggressive tumours and a shorter survival than those with only *KRAS* mutant cancers (Ji *et al*, 2007). In our study, we were not able to detect a significant survival difference for patients whose tumours contained *LKB1* mutations alone or concurrently with *KRAS* mutations (data not shown) likely because of the limited number of tumour specimens. Additional studies are needed to clarify the prognostic impact of *LKB1* mutations in humans with NSCLC. In the present study ~2 out of 3 of *LKB1* mutant tumours were *KRAS* wild type (Table 3). One possibility is that such tumours contain a concurrent mutation in another oncogene that activates the same signalling pathway as *KRAS*. For this reason, we examined our tumours for *BRAF* mutations, which are found in 1–2% of NSCLC (Naoki *et al*, 2002). We detected a concurrent *LKB1* mutation in one of the four *BRAF* mutant tumours (Table 3). This tumour was wild type for *KRAS* (data not shown). In addition, some of the *BRAF* mutant NSCLC cell lines (NCI-H1395, G469A; NCI-H1666, G466V) also contain a concurrent *LKB1* mutation (Sanchez-Cespedes *et al*, 2002; Bamford *et al*, 2004; Carretero *et al*, 2004). Future studies will help further clarify whether *LKB1* mutations occur concurrently with other genomic alterations in NSCLC and the impact of this on patient outcome.

Our study employed a mutation scanning technology to screen for *LKB1* mutations at the cDNA level (Janne *et al*, 2006). This was advantageous as the entire coding region of *LKB1* could be rapidly screened for a mutation using just two overlapping cDNA fragments. *LKB1* is a challenging gene to analyse at the genomic DNA level because of its high guanine–cytosine content. In addition, as many of the *LKB1* mutations are small deletions (Table 2) or involve deletions of entire exons, these would be missed using exon-specific genome sequencing methods. Our method, however, does have limitations as it would miss deletions at the site of PCR primers, whole gene deletions, or deletions within the promoter region all of which have been infrequently detected in PJS (Volikos *et al*, 2006). Thus our studies may underestimate the true *LKB1* mutation frequency in NSCLC. In addition, our method is limited to the analysis of fresh tumour specimens, which are available only from the minority of NSCLC patients. Furthermore, as techniques isolating mRNA from formalin-fixed paraffin-embedded tumour specimens continue to improve, this rapid mutation scanning technique can be used to analyse broader populations of tumours from NSCLC patients. Future studies may need to employ a combination of *LKB1* mutation detection methodologies including the current method, MLPA and direct sequencing.

ACKNOWLEDGEMENTS

This study is supported by grants from the National Institutes of Health 1RO1CA114465-01 (BYY, BEJ, and PAJ), the National Cancer Institute Lung SPORE P20CA90578-02 (BYY and BEJ), American Cancer Society RSG-06-102-01-CCE (BYY and PAJ), Finnish Medical Foundation (JPK), Finnish Cultural Foundation (JPK), and Academy of Finland (JPK). PAJ, MM, and BEJ are part of a pending patent application on *EGFR* mutations.

REFERENCES

Abed AA, Gunther K, Kraus C, Hohenberger W, Ballhausen WG (2001) Mutation screening at the RNA level of the STK11/LKB1 gene in Peutz-Jeghers syndrome reveals complex splicing abnormalities and a novel mRNA isoform (STK11 c.597(insertion mark)598insIVS4). *Hum Mutat* **18:** 397–410

Ahrendt SA, Decker PA, Alawi EA, Zhu Yr YR, Sanchez-Cespedes M, Yang SC, Haasler GB, Kajdacsy-Balla A, Demeure MJ, Sidransky D (2001) Cigarette smoking is strongly associated with mutation of the K-ras gene in patients with primary adenocarcinoma of the lung. *Cancer* **92:** 1525–1530

Avizienyte E, Loukola A, Roth S, Hemminki A, Tarkkanen M, Salovaara R, Arola J, Butzow R, Husgafvel-Pursiainen K, Kokkola A, Jarvinen H, Aaltonen LA (1999) LKB1 somatic mutations in sporadic tumors. *Am J Pathol* **154:** 677–681

Avizienyte E, Roth S, Loukola A, Hemminki A, Lothe RA, Stenwig AE, Fossa SD, Salovaara R, Aaltonen LA (1998) Somatic mutations in LKB1 are rare in sporadic colorectal and testicular tumors. *Cancer Res* **58:** 2087–2090

Ballhausen WG, Gunther K (2003) Genetic screening for Peutz-Jeghers syndrome. *Expert Rev Mol Diagn* **3:** 471–479

Bamford S, Dawson E, Forbes S, Clements J, Pettett R, Dogan A, Flanagan A, Teague J, Futreal PA, Stratton MR, Wooster R (2004) The COSMIC (Catalogue of Somatic Mutations in Cancer) database and website. *Br J Cancer* **91:** 355–358

Bhattacharjee A, Richards WG, Staunton J, Li C, Monti S, Vasa P, Ladd C, Beheshti J, Bueno R, Gillette M, Loda M, Weber G, Mark EJ, Lander ES, Wong W, Johnson BE, Golub TR, Sugarbaker DJ, Meyerson M (2001) Classification of human lung carcinomas by mRNA expression profiling reveals distinct adenocarcinoma subclasses. *Proc Natl Acad Sci USA* **98:** 13790–13795

Breslow NE, Day NE, International Agency for Research on Cancer (1980) Statistical methods in cancer research. Vol. 1. Analysis of case-control studies. IARC scientific publications; no. 32. International Agency for Research on Cancer: Lyon

Carretero J, Medina PP, Pio R, Montuenga LM, Sanchez-Cespedes M (2004) Novel and natural knockout lung cancer cell lines for the LKB1/STK11 tumor suppressor gene. *Oncogene* **23:** 4037–4040

Engelman JA, Mukohara T, Zejnullahu K, Lifshits E, Borras AM, Gale CM, Naumov GN, Yeap BY, Jarrell E, Sun J, Tracy S, Zhao X, Heymach JV, Johnson BE, Cantley LC, Janne PA (2006) Allelic dilution obscures detection of a biologically significant resistance mutation in EGFR-amplified lung cancer. *J Clin Invest* **116:** 2695–2706

Fernandez P, Carretero J, Medina PP, Jimenez AI, Rodriguez-Perales S, Paz MF, Cigudosa JC, Esteller M, Lombardia L, Morente M, Sanchez-Verde L, Sotelo T, Sanchez-Cespedes M (2004) Distinctive gene expression of human lung adenocarcinomas carrying LKB1 mutations. *Oncogene* **23:** 5084–5091

Hayes DN, Monti S, Parmigiani G, Gilks CB, Naoki K, Bhattacharjee A, Socinski MA, Perou C, Meyerson M (2006) Gene expression profiling reveals reproducible human lung adenocarcinoma subtypes in multiple independent patient cohorts. *J Clin Oncol* **24:** 5079–5090

Hearle N, Schumacher V, Menko FH, Olschwang S, Boardman LA, Gille JJ, Keller JJ, Westerman AM, Scott RJ, Lim W, Trimbath JD, Giardiello FM, Gruber SB, Offerhaus GJ, de Rooij FW, Wilson JH, Hansmann A, Moslein G, Royer-Pokora B, Vogel T, Phillips RK, Spigelman AD, Houlston RS (2006) Frequency and spectrum of cancers in the Peutz-Jeghers syndrome. *Clin Cancer Res* **12:** 3209–3215

Hemminki A, Markie D, Tomlinson I, Avizienyte E, Roth S, Loukola A, Bignell G, Warren W, Aminoff M, Hoglund P, Jarvinen H, Kristo P, Pelin K, Ridanpaa M, Salovaara R, Toro T, Bodmer W, Olschwang S, Olsen AS, Stratton MR, de la Chapelle A, Aaltonen LA (1998) A serine/threonine kinase gene defective in Peutz-Jeghers syndrome. *Nature* **391:** 184–187

Janne PA, Borras AM, Kuang Y, Rogers AM, Joshi VA, Liyanage H, Lindeman N, Lee JC, Halmos B, Maher EA, Distel RJ, Meyerson M, Johnson BE (2006) A rapid and sensitive enzymatic method for epidermal growth factor receptor mutation screening. *Clin Cancer Res* **12:** 751–758

Janne PA, Johnson BE (2006) Effect of epidermal growth factor receptor tyrosine kinase domain mutations on the outcome of patients with non-small cell lung cancer treated with epidermal growth factor receptor tyrosine kinase inhibitors. *Clin Cancer Res* **12:** 4416s–4420s

Ji H, Ramsey MR, Hayes DN, Fan C, McNamara K, Kozlowski P, Torrice C, Wu MC, Shimamura T, Perera SA, Liang MC, Cai D, Naumov GN, Bao L, Contreras CM, Li D, Chen L, Krishnamurthy J, Koivunen J, Chirieac LR, Padera RF, Bronson RT, Lindeman NI, Christiani DC, Lin X, Shapiro GI, Janne PA, Johnson BE, Meyerson M, Kwiatkowski DJ, Castrillon DH, Bardeesy N, Sharpless NE, Wong KK (2007) LKB1 modulates lung cancer differentiation and metastasis. *Nature* **448:** 807–810

Kosaka T, Yatabe Y, Endoh H, Kuwano H, Takahashi T, Mitsudomi T (2004) Mutations of the epidermal growth factor receptor gene in lung cancer: biological and clinical implications. *Cancer Res* **64:** 8919–8923

Launonen V (2005) Mutations in the human LKB1/STK11 gene. *Hum Mutat* **26:** 291–297

Launonen V, Avizienyte E, Loukola A, Laiho P, Salovaara R, Jarvinen H, Mecklin JP, Oku A, Shimane M, Kim HC, Kim JC, Nezu J, Aaltonen LA (2000) No evidence of Peutz-Jeghers syndrome gene LKB1 involvement in left-sided colorectal carcinomas. *Cancer Res* **60:** 546–548

Marchetti A, Martella C, Felicioni L, Barassi F, Salvatore S, Chella A, Camplese PP, Iarussi T, Mucilli F, Mezzetti A, Cuccurullo F, Sacco R, Buttitta F (2005) EGFR mutations in non-small-cell lung cancer: analysis of a large series of cases and development of a rapid and sensitive method for diagnostic screening with potential implications on pharmacologic treatment. *J Clin Oncol* **23:** 857–865

Matsumoto S, Iwakawa R, Takahashi K, Kohno T, Nakanishi Y, Matsuno Y, Suzuki K, Nakamoto M, Shimizu E, Minna JD, Yokota J (2007) Prevalence and specificity of LKB1 genetic alterations in lung cancers. *Oncogene* **26:** 5911–5918

Naoki K, Chen TH, Richards WG, Sugarbaker DJ, Meyerson M (2002) Missense mutations of the BRAF gene in human lung adenocarcinoma. *Cancer Res* **62:** 7001–7003

Ono M, Hirata A, Kometani T, Miyagawa M, Ueda S, Kinoshita H, Fujii T, Kuwano M (2004) Sensitivity to gefitinib (Iressa, ZD1839) in non-small cell lung cancer cell lines correlates with dependence on the epidermal growth factor (EGF) receptor/extracellular signal-regulated kinase 1/2 and EGF receptor/Akt pathway for proliferation. *Mol Cancer Ther* **3:** 465–472

Onozato R, Kosaka T, Achiwa H, Kuwano H, Takahashi T, Yatabe Y, Mitsudomi T (2007) LKB1 gene mutations in Japanese lung cancer patients. *Cancer Sci* **98:** 174–175

Pham D, Kris MG, Riely GJ, Sarkaria IS, McDonough T, Chuai S, Venkatraman ES, Miller VA, Ladanyi M, Pao W, Wilson RK, Singh B, Rusch VW (2006) Use of cigarette-smoking history to estimate the likelihood of mutations in epidermal growth factor receptor gene exons 19 and 21 in lung adenocarcinomas. *J Clin Oncol* **24:** 1700–1704

Sanchez-Cespedes M, Parrella P, Esteller M, Nomoto S, Trink B, Engles JM, Westra WH, Herman JG, Sidransky D (2002) Inactivation of LKB1/STK11 is a common event in adenocarcinomas of the lung. *Cancer Res* **62:** 3659–3662

Shaw RJ, Bardeesy N, Manning BD, Lopez L, Kosmatka M, DePinho RA, Cantley LC (2004) The LKB1 tumor suppressor negatively regulates mTOR signaling. *Cancer Cell* **6:** 91–99

Tiainen M, Ylikorkala A, Makela TP (1999) Growth suppression by Lkb1 is mediated by a G(1) cell cycle arrest. *Proc Natl Acad Sci USA* **96:** 9248–9251

Tracy S, Mukohara T, Hansen M, Meyerson M, Johnson BE, Janne PA (2004) Gefitinib induces apoptosis in the EGFRL858R non-small-cell lung cancer cell line H3255. *Cancer Res* **64:** 7241–7244

Volikos E, Robinson J, Aittomaki K, Mecklin JP, Jarvinen H, Westerman AM, de Rooji FW, Vogel T, Moeslein G, Launonen V, Tomlinson IP, Silver AR, Aaltonen LA (2006) LKB1 exonic and whole gene deletions are a common cause of Peutz-Jeghers syndrome. *J Med Genet* **43:** e18

Interstitial Lung Disease in Japanese Patients with Lung Cancer

A Cohort and Nested Case-Control Study

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Rationale: **Interstitial lung disease (ILD) occurs in Japanese patients with non–small cell lung cancer (NSCLC) receiving gefitinib.**

Objectives: **To elucidate risk factors for ILD in Japanese patients with NSCLC during treatment with gefitinib or chemotherapy.**

Methods: **In a prospective epidemiologic cohort, 3,166 Japanese patients with advanced/recurrent NSCLC were followed for 12 weeks on 250 mg gefitinib (n = 1,872 treatment periods) or chemotherapy (n = 2,551). Patients who developed acute ILD (n = 122) and randomly selected control subjects (n = 574) entered a case-control study. Adjusted incidence rate ratios were estimated from case-control data by odds ratios (ORs) with 95% confidence intervals (CIs) using logistic regression. Crude (observed) incidence rates and risks were calculated from cohort data.**

Measurements and Main Results: **The observed (unadjusted) incidence rate over 12 weeks was 2.8 (95% CI, 2.3–3.3) per 1,000 person-weeks, 4.5 (3.5–5.4) for gefitinib versus 1.7 (1.2–2.2) for chemotherapy; the corresponding observed naive cumulative incidence rates at the end of 12-week follow-up were 4.0% (3.0–5.1%) and 2.1% (1.5–2.9%), respectively. Adjusted for imbalances in risk factors between treatments, the overall OR for gefitinib versus chemotherapy was 3.2 (1.9–5.4), elevated chiefly during the first 4 weeks (3.8 [1.9–7.7]). Other ILD risk factors in both groups included the following: older age, poor World Health Organization performance status, smoking, recent NSCLC diagnosis, reduced normal lung on computed tomography scan, preexisting chronic ILD, concurrent cardiac disease. ILD-related deaths in patients with ILD were 31.6% (gefitinib) versus 27.9% (chemotherapy); adjusted OR, 1.05 (95% CI, 0.3–3.2).**

Conclusions: **ILD was relatively common in these Japanese patients with NSCLC during therapy with gefitinib or chemotherapy, being higher in the older, smoking patient with preexisting ILD or poor performance status. The risk of developing ILD was higher with gefitinib than chemotherapy, mainly in the first 4 weeks.**

Keywords: non–small cell lung cancer; interstitial lung disease; Japanese patients; gefitinib, chemotherapy

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

Acute interstitial lung disease (ILD) occurs in Japanese patients with non–small cell lung cancer (NSCLC) receiving gefitinib. There is, however, limited knowledge about risk factors for ILD and the incidence of ILD in patients with NSCLC receiving other treatments.

What This Study Adds to the Field

Acute ILD was common in Japanese patients with NSCLC receiving chemotherapy or gefitinib, with higher risk for gefitinib. Age, performance status, smoking, and preexisting chronic ILD were also important risk factors, aiding clinicians in treatment selection.

Epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors are a well-established therapy for the treatment of non–small cell lung cancer (NSCLC) in many countries. They are generally well tolerated and not typically associated with the cytotoxic side effects commonly seen with chemotherapy.

The EGFR tyrosine kinase inhibitor gefitinib (IRESSA; AstraZeneca, London, U.K.) was first approved for the treatment of advanced NSCLC in Japan in July 2002. In clinical trials and in preapproval compassionate clinical use, some reports of interstitial lung disease (ILD)–type events had been observed. As the drug was made more widely available in Japan after approval, however, an increasing number of spontaneous reports for ILD appeared.

ILD is a disease that affects the parenchyma or alveolar region of the lungs (1). When associated with drug use, it can present precipitously with acute diffuse alveolar damage, which is fatal in some patients (2). Chest imaging shows ground-glass density and patients present with severe breathlessness. There is no specific treatment, but supportive therapy including oxygen, corticosteroids, or assisted ventilation is indicated. Acute exacerbations of ILD have previously been considered relatively rare in many settings, with Japan as a notable exception (3), but recent studies of patients with idiopathic pulmonary fibrosis (IPF) have challenged this and underlined this important risk (4).

ILD, especially IPF, is a known comorbidity in patients with NSCLC and has also been associated with many other lung cancer therapies (5). Rates of acute ILD events up to and exceeding 10% have been reported in patients receiving chemotherapy and radiotherapy (6–11). It is recognized that ILD is more common in Japan than elsewhere (5, 6, 12, 13).

(Received in original form October 11, 2007; accepted in final form March 12, 2008)

Supported by AstraZeneca.

*A list of Japan Thoracic Radiology Group members may be found in the ACKNOWLEDGMENT.

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This article has an online supplement, which is accessible from this issue's table of contents at www.atsjournals.org

Am J Respir Crit Care Med Vol 177. pp 1348–1357, 2008
Originally Published in Press as DOI: 10.1164/rccm.200710-1501OC on March 12, 2008
Internet address: www.atsjournals.org

When safety reports of acute ILD-type events in gefitinib-treated patients appeared in Japan, there was limited knowledge about ILD in patients with NSCLC. There was a need to better understand baseline incidence on different treatments, risk factors for developing ILD, and whether gefitinib might be associated with increased risk of ILD, or if patient selection or other aspects were involved. A pharmacoepidemiologic study was designed and conducted by an independent academic team together with scientists from AstraZeneca to define the risk and increase understanding of ILD in Japanese patients with NSCLC. Some of the results of this study have been previously reported in the form of conference abstracts (14, 15).

METHODS

See also the online supplement for further details on methods.

Overall Study Design

A nonrandomized cohort study with a nested case-control study component was conducted between November 12, 2003, and February 22, 2006, in 50 centers across Japan. Patients with advanced or recurring NSCLC who had received at least one chemotherapy regimen were eligible for cohort entry. Patients and their physicians selected the most appropriate treatment (gefitinib 250 mg or chemotherapy) and the patients were followed for up to 12 weeks after treatment initiation. Basic data were collected at the start of follow-up and included sex, age, World Health Organization (WHO) performance status (PS), and tumor histology. If a patient switched to a new treatment, he or she could be re-enrolled for a new treatment period of 12 weeks, provided he or she was still eligible.

Patients who developed acute ILD events during the follow-up were registered to the case-control study nested within the cohort as clinically diagnosed potential cases. For each potential case, four patients who had not yet developed ILD were randomly selected as appropriate control subjects from patients registered to the cohort at that time, and extensive clinical and demographic risk factor data were collected on cases and control subjects (*see* Figure E1 in the online supplement).

The study followed Good Clinical Practice procedures. An independent external epidemiology advisory board provided advice on design, conduct, and analysis of the study.

Diagnosis of ILD

To ensure an accurate diagnosis of ILD, several study design components were implemented: (*1*) an information card to all cohort patients, alerting them to the symptoms of ILD; (*2*) internationally agreed criteria for the diagnosis of ILD and a diagnostic algorithm (*see* Figure E2) developed from the American Thoracic Society/European Respiratory Society consensus statement (1); and (*3*) a blinded diagnostic review of all clinically diagnosed potential ILD cases registered to the study by an independent case review board (CRB) of radiologists and clinicians.

Evaluation of Preexisting Lung Conditions

The CRB also blindly evaluated pretreatment computed tomography (CT) scans for the presence of a number of pulmonary conditions: preexisting (chronic) ILD (mainly IPF), drug-induced lung disease, pulmonary emphysema, radiation pneumonitis, lymphangitis carcinomatosis, and healed tuberculosis, and evaluated the extent of normal lung, as well as the extent of areas adherent to pleura.

Detailed Data Collection

For cases and control subjects, detailed data on NSCLC treatment, demography, cancer histology, clinical stage and the presence of metastases, WHO PS, smoking, previous cancer treatments, past and current medical history, surgical history, and concomitant medication and therapy were collected. Data on serious adverse events (SAEs) and hence all-cause mortality were collected for the gefitinib-treated patients in the cohort only; thus, information on mortality from causes other than ILD in chemotherapy-treated patients is not available from this study.

Statistical Analysis

From cohort data, we estimated observed person-time incidence rates as well as two measures of the observed "risk" of acute ILD to a patient; a naive estimate of observed cumulative incidence (incidence proportion, "frequency"), and risk up to 84 days by the Kaplan-Meier method.

Control subjects for the nested case-control study were sampled using incidence density sampling, and consequently the odds ratio (OR) obtained from the case-control analysis estimates the study incidence rate ratio (and approximately estimates the risk ratio) (16).

For the case-control statistical analysis, it was initially verified that the convenience matching for calendar time implicit in the risk set control sampling could be disregarded. In tabular analyses, we then identified potential confounders and risk factors, using as selection criteria a 10% change in the OR estimate for gefitinib versus chemotherapy treatment when stratifying for each factor separately, and a risk factor crude OR of less than 0.5 or more than 2.0, respectively. We also identified potential interactions between treatment and other risk factors, or between two potential risk factors. Modeling using logistic regression then proceeded in the corresponding four steps. Few previous data were available on risk factors for ILD in patients with NSCLC and so a hypothesis-free stepwise process with loose P value criteria ($P < 0.20$) for selection was used throughout to avoid bias.

Two sensitivity analyses were performed. First, to investigate the potential influence of the modeling approach used, a propensity score analysis was performed (17). This analysis provides an alternative way of adjusting for potential confounding bias by stratifying for a compound score based on predictors of treatment (*see* online supplement for details). Second, we estimated the possible bias due to misclassification of disease under reasonable assumptions of diagnostic error.

ILD-related mortality among the patients who developed acute ILD on gefitinib or chemotherapy treatment was obtained. Modeling of risk factors for ILD-related mortality followed a similar process to the ILD risk factor modeling. For gefitinib-treated patients, two additional data items were available: total all-cause mortality, which was analyzed by the Kaplan-Meier method, and SAEs, for which frequencies and possible consequences in terms of treatment discontinuation and death were calculated.

RESULTS

Cohort Subjects and Treatments

Cohort participation rates were high. In 10 sampled study centers, 89.6% of eligible patients were enrolled to the cohort. The number of treatment periods and subjects are summarized in Table 1. In total, 4,423 treatment periods in 3,159 subjects were available for analysis. In the cohort, 70.8% of patients had only one treatment period, 21.5% had two periods, and the remaining 7.8% of patients had three or more treatment periods registered (Table 1). Chemotherapy included a wide range of treatments, the most common being taxane monotherapy, followed by taxane+platinum and gemcitabine+vinorelbine combinations.

Cases and Control Subjects

In the overall cohort data of all treatment periods, clinicians reported 155 suspected cases of acute ILD during the follow-up, of which 122 were confirmed by the CRB after blinded review of CT and clinical data—79 of 103 gefitinib-treated (76.7%) and 43 of 52 chemotherapy-treated (82.7%) subjects. A total of 574 eligible control subjects were sampled from the person-time of the cohort. Almost all ILD cases and selected control subjects consented to participate in the nested case-control study, with final participation rates of 98.1 and 92.0%, respectively. Valid data from the CRB review of CT scans were available for 115 cases and 520 control subjects.

Descriptive Data

On data items available for the full cohort (sex, age, WHO PS, and tumor histology), the control subjects were quite represen-

TABLE 1. NUMBER OF TREATMENT PERIODS AND SUBJECTS IN THE COHORT AND NUMBER OF CASES AND CONTROLS IN THE NESTED CASE-CONTROL STUDY

	Gefitinib (*n*)	Chemotherapy (*n*)	Total (*n*)
Treatment periods registered to cohort	1,901	2,572	4,473
No treatment administered	9	15	24
Ineligible subjects	6	6	12
Protocol deviations	14	0	14
Per-protocol study cohort (treatment periods)	1,872	2,551	4,423
Subjects in cohort (first treatment periods)*	1,489	1,677	3,166
No. of subjects and order of treatment periods registered to the cohort			
1 treatment period: G			1,199
1 treatment period: C			1,036
2 treatment periods: GC			194
2 treatment periods: CG			248
2 treatment periods: CC			228
2 treatment periods: GG			9
3–8 treatment periods†: initial G			81
3–9 treatment periods‡: initial C			166
First gefitinib treatment periods total§	1,849		
Confirmed cases‖	79	43	122
Rejected cases‖	24	9	33
Control subjects	252	322	574

Definition of abbreviations: C = chemotherapy; G = gefitinib.

* Counts the first registered treatment period for each subject.

† 70% of these with three periods.

‡ 78% of these with three periods.

§ Counts the first gefitinib treatment period for all subjects with one or more gefitinib treatment registrations to the cohort; also when their very first registration was for chemotherapy.

‖ Cases registered by clinical investigators to the case-control study and subsequently confirmed or rejected by the case review board (blinded review of case diagnostic data).

tative of the overall cohort (details not shown). Comparisons of the gefitinib- and chemotherapy-treated control groups as representative of the cohort indicated that the former included more women, never-smokers, adenocarcinoma tumors, and poorer PS, as well as less preexisting ILD and pulmonary emphysema on CT scan (Tables 2 and 3). ILD cases, regardless of treatment, were more likely than cohort control subjects to be older, male, smokers, with squamous cell carcinoma histology, and have poor PS (Tables 2 and 3). The frequency of preexisting ILD and pulmonary emphysema was higher in cases, reflected also in a lower extent of normal lung on CT scan.

Cohort Analysis of ILD Occurrence

The observed incidence rate of acute ILD over the entire 12-week follow-up in the overall cohort was 2.8 per 1,000 person-weeks—4.5 in the gefitinib-treated and 1.7 in the chemotherapy subcohort (Table 4). The observed incidence in the gefitinib-treated subcohort was highest in the first 4 weeks after starting treatment, greater than in the chemotherapy-treated subcohort. In the following two 4-week periods, the incidence was lower with no clear difference (Table 4, Figure 1A). The naive cumulative incidence of ILD at 84 days (i.e., observed frequency or proportion of the original cohort that developed ILD in the study) for patients in their first study treatment period was 4.0 and 2.1% for gefitinib- and chemotherapy-treated patients, respectively (Table 4), whereas the estimated theoretical 12-week risk of ILD (i.e., taking competing causes of death and loss to follow-up into consideration; Kaplan-Meier method) was 4.5 and 2.4%, respectively (Table 4, Figure 1B). Thus, the observed cohort rates and risks suggested an association of increased ILD occurrence with gefitinib treatment mainly in the first 4 weeks after treatment initiation. All cohort estimates are unadjusted for imbalances between treatments in other risk factors. Detailed comparisons between the treatments therefore used the adjusted case-control OR (as an estimate of the adjusted incidence rate ratio) to achieve comparability.

Case-Control Analysis of ILD Occurrence and Risk Factors

Major results. The OR of developing acute ILD with gefitinib treatment versus chemotherapy, adjusted for the full predictor model of major confounders together with additional identified important risk factors and interactions, was 3.2 (95% confidence interval [CI], 1.9–5.4) (Table 5). Several risk factors aside from treatment also had strong effects, including WHO PS, as well as smoking status and preexisting ILD together with the extent of normal lung on CT scan, which interacted in a complex way in the model (Table 5, Figure 2). Preexisting ILD was confirmed as a strong risk factor, with OR point estimates ranging from 4.8 to 25.3 depending on the extent of remaining normal lung on CT scan, in comparison with patients without preexisting ILD and high extent of normal lung on CT scan (Table 5). The full set of ILD risk factors in both groups from the final model thus included older age (≥55 yr), WHO PS (≥2), smoking, short duration since NSCLC diagnosis (<6 mo), reduced extent of normal lung on CT scan (<50%), preexisting ILD, and concurrent cardiac disease. Although some potential significant interactions were seen in the initial tabular analyses (Table E1), no significant interactions with treatment (i.e., treatment-specific risk factors, or variation in treatment-related effect in subgroups defined by another risk factor) were identified in the modeling after adjustment for the relevant risk factors.

When the case-control analyses focused on the first 4 weeks after treatment initiation (because the unadjusted cohort analyses above indicated that the bulk of the association with gefitinib appeared to be for this time interval) the estimated OR adjusted for a full predictor model developed on this period's data was 3.8 (95% CI, 1.9–7.7). The same model produced an OR for Weeks 5–8 of 1.6 (95% CI, 0.5–4.8), whereas the final 4-week period had too few cases for an adequate estimate. The estimate for Weeks 5–12 combined, using this same model, was 2.5 (95% CI, 1.1–5.8). The important covariates and predictors were the same in this model as in the model for the full 12-week data, with the exception of age, preexisting cardiac disease, and preexisting pulmonary emphysema, which were not included. Due to sparse data beyond 4 weeks, independent models for Weeks 5–8, 9–12, and 5–12 could not be developed.

Confounding and sensitivity analysis. In the overall 12-week basic analysis, moderately strong confounding by other risk factors was found. The crude OR of developing ILD with gefitinib treatment versus chemotherapy was 2.3 (95% CI, 1.5–3.6). When adjusted for some of the most important potential confounders one at a time, the adjusted OR point estimate for the association of treatment with ILD occurrence ranged from 2.1 to 3.1 (*see* Table E1 for details). The most important confounder was severity of preexisting ILD with strong negative confounding, and the only one that resulted in a lower adjusted OR than 2.3 (positive confounding) was WHO PS.

The propensity score analysis approach identified the following variables as the most important predictors of selecting gefitinib treatment in this study: female sex; nonsmoking status; non–squamous tumor histology; poor PS; preexisting lymphangitis carcinomatosis; no previous gefitinib treatment; and no preexisting ILD, emphysema, or radiation pneumonitis. The

TABLE 2. CHARACTERISTICS OF CONFIRMED CASES AND CONTROL SUBJECTS (AS A RANDOM SAMPLE OF THE STUDY COHORT)

	Cases (*n* = 122)	Controls (*n* = 574)	Gefitinib Control Sample (*n* = 252)	Chemotherapy Control Sample (*n* = 322)
Sex				
Male	92 (75.4)	360 (62.7)	126 (50.0)	234 (72.7)
Female	30 (24.6)	214 (37.3)	126 (50.0)	88 (27.3)
Age				
<55 yr	11 (9.0)	95 (16.6)	43 (17.1)	52 (16.1)
≥55 yr	111 (91.0)	479 (83.4)	209 (82.9)	270 (83.9)
WHO performance status				
0	18 (14.8)	154 (26.8)	68 (27.0)	86 (26.7)
1	69 (56.6)	358 (62.4)	148 (58.7)	210 (65.2)
2–3	35 (28.7)	62 (10.8)	36 (14.3)	26 (8.1)
Histologic type				
Squamous cell carcinoma	29 (23.8)	103 (17.9)	27 (10.7)	76 (23.6)
Adenocarcinoma	80 (65.6)	414 (72.1)	207 (82.1)	207 (64.3)
Others	13 (10.7)	57 (9.9)	18 (7.1)	39 (12.1)
Smoking history				
No	21 (17.2)	192 (33.4)	113 (44.8)	79 (24.5)
Yes	100 (82.0)	382 (66.6)	139 (55.2)	243 (75.5)
Unknown	1 (0.8)	0 (0.0)	0 (0.0)	0 (0.0)
Time since diagnosis of NSCLC				
<0.5 yr	49 (40.2)	153 (26.7)	65 (25.8)	88 (27.3)
0.5 to <1 yr	36 (29.5)	154 (26.8)	67 (26.6)	87 (27.0)
≥1 yr	37 (30.3)	267 (46.5)	120 (47.6)	147 (45.7)
Previous gefitinib treatment				
No	113 (92.6)	465 (81.0)	241 (95.6)	224 (69.6)
Yes	9 (7.4)	109 (19.0)	11 (4.4)	98 (30.4)
Concurrent cardiac disease				
No	111 (91.0)	556 (96.7)	244 (96.4)	312 (96.9)
Yes	11 (9.0)	19 (3.3)	9 (3.6)	10 (3.1)

Definition of abbreviations: NSCLC = non–small cell lung cancer; WHO = World Health Organization.
Values shown are numbers (%).

estimated OR of developing ILD for gefitinib treatment when stratifying by the propensity score was 3.3 (95% CI, 1.9–5.5), very similar to the primary result, suggesting that the primary regression modeling approach well captured the confounding in the data.

If some misclassification of ILD diagnosis remains despite the design features aimed to minimize it, the adjusted OR point estimate of 3.2 may apart from random variation be subject to systematic bias. A sensitivity analysis to evaluate the possible magnitude of such bias due to misclassification of ILD diagnosis suggested that the true study point estimate for the adjusted OR would be expected to lie between 2.6 and 4.8, assuming diagnostic sensitivity of more than 80% for both gefitinib- and chemotherapy-treated patients, and specificity of more than 99.0% for gefitinib and more than 99.5% for chemotherapy. Lower values for sensitivity/specificity were considered very unlikely for this serious condition in a cancer patient population, in this study setting.

Analysis of ILD Mortality

Mortality due to ILD among gefitinib- or chemotherapy-treated patients. The mortality due to ILD for the patients who developed acute ILD was 31.6% (95% CI, 21.6–43.1) among gefitinib-treated patients and 27.9% (95% CI, 15.3–43.7) among those with other treatments; the OR was 1.05 (95% CI, 0.3–3.2) for gefitinib versus chemotherapy, adjusted for relevant risk factors. Several other factors were strong predictors of a fatal outcome for patients with ILD, including age of 65 years or older, smoking history, preexisting ILD, CT scan evidence of reduced normal lung (≤50%), and/or extensive areas adherent to pleura (≥50%), with ORs ranging from 2.4 to 11.7 (*see* Table E2).

Overall mortality among gefitinib-treated patients. In the gefitinib-treated cohort in whom such data were available, an analysis of mortality from all causes by the Kaplan-Meier method showed that cumulative mortality at 12 weeks among the patients who did develop ILD was 58.7%, compared with 14.6% (95% CI, 12.8–16.3) among the large majority who did not develop ILD (Figure 3). For the entire gefitinib cohort, including the subjects who developed ILD, the observed cumulative mortality was 16.0% (95% CI, 14.3–17.8), so that the increased mortality in ILD cases impacted the total survival rate at 12 weeks in the overall gefitinib-treated cohort only to a limited extent, reducing survival from 85.4 to 84.0%.

SAEs among Gefitinib-treated Patients

SAEs were only collected for gefitinib-treated patients in the cohort, and a total of 198 patient registrations reported SAEs (10.5%), of which 38 (2.0%) reported SAEs resulting in a fatal outcome. Within this group, there were 142 patient registrations with drug-related (as reported by the physicians) SAEs (7.5%), of which 30 (1.6%) resulted in a fatal outcome. The majority of these (25 out of 30) were due to ILD-type events. There were 122 patient registrations where study treatment was discontinued due to the reported SAEs (6.5%). SAEs seen in the gefitinib-treated patients were generally consistent with the known safety profile of gefitinib and/or the patient's underlying disease and comorbidities.

DISCUSSION

This study provides important information on ILD in an advanced/recurrent NSCLC setting in Japanese patients in Japan, and it is the largest prospective study of this condition

TABLE 3. CHARACTERISTICS OF CONFIRMED CASES AND CONTROLS (AS A REPRESENTATIVE SAMPLE OF THE STUDY COHORT)

	Cases ($n = 115$)	Controls ($n = 520$)	Gefitinib Control Sample ($n = 240$)	Chemotherapy Control Sample ($n = 280$)
Severity of preexisting interstitial lung disease on CT scan (CRB evaluation)				
No ILD	84 (73.0)	473 (91.0)	231 (96.3)	242 (86.4)
Mild	15 (13.0)	28 (5.4)	8 (3.3)	20 (7.1)
Moderate	12 (10.4)	14 (2.7)	1 (0.4)	13 (4.6)
Severe	4 (3.5)	5 (1.0)	0 (0.0)	5 (1.8)
Severity of preexisting pulmonary emphysema on CT scan (CRB evaluation)				
No emphysema	56 (48.7)	326 (62.8)	176 (73.3)	150 (53.8)
Mild	35 (30.4)	92 (17.7)	36 (15.0)	56 (20.1)
Moderate	18 (15.7)	59 (11.4)	16 (6.7)	43 (15.4)
Severe	6 (5.2)	42 (8.1)	12 (5.0)	30 (10.8)
Extent of normal lung on CT scan (CRB evaluation)				
Low (10–50%)	49 (42.6)	133 (25.6)	56 (23.3)	77 (27.5)
Normal (60–100%)	66 (57.4)	387 (74.4)	184 (76.7)	203 (72.5)

Definition of abbreviations: CRB = case review board; ILD = interstitial lung disease.
Values shown are numbers (%) of total subjects with available CRB data.

to date. For the first time, the risk of acute ILD events for a large and relatively unselected chemotherapy-treated NSCLC patient cohort in Japan was determined in clinical practice. The study also quantified the greater risk of developing acute ILD associated with gefitinib treatment than with conventional chemotherapy, mainly in the first 4 weeks after treatment initiation. The study confirmed and further defined risk factors for developing ILD with gefitinib or chemotherapy. The factors included older age, poor WHO PS, smoking, short duration since diagnosis of NSCLC, reduced normal lung on CT scan, preexisting ILD, and concurrent cardiac disease. Several of these factors, or related factors, had been reported previously in bivariate or multivariate analyses from other studies (8, 18, 19). These risk factors were the same for patients treated with gefitinib or chemotherapy in the study, and no treatment-specific risk factors were identified. In particular, patients with CT evidence of preexisting ILD (chronic) were at considerably elevated risk of developing acute ILD during treatment, but there were relatively few subjects with preexisting ILD and the data did not indicate a statistically significant difference in treatment-related risk depending on the preexisting ILD status. Of clinical relevance, some of these risk factors were just as strong as, or stronger than, gefitinib treatment, for example having a poor WHO PS (≥2) rather than a good PS (OR, 4.0; 95% CI, 1.85–8.75), implying that they can be used to identify patients at particular risk of ILD in clinical practice. The relationship between ILD and pharmacokinetic characteristics of gefitinib, as well as genetic polymorphisms and proteomics determined in

TABLE 4. MEASURES OF DISEASE OCCURRENCE FOR ACUTE INTERSTITIAL LUNG DISEASE ESTIMATED FROM THE COHORT DATA (INCIDENCE RATE, CUMULATIVE INCIDENCE)

	Gefitinib Cohort	Chemotherapy Cohort
Overall observed incidence rate 0–84 d		
No. of treatment periods at Day 0	1,872	2,551
Cases of ILD/person-weeks	79/17,740	43/25,224
Incidence rate per week (95% CI)	0.00445 (0.00347–0.00544)	0.00170 (0.00120–0.00221)
Overall observed incidence rate 0–28 d		
No. of treatment periods at Day 0	1,872	2,551
Cases of ILD / person-weeks	56/7,032	21/9,902
Incidence rate per week (95% CI)	0.00796 (0.00588–0.01005)	0.00212 (0.00121–0.00303)
Overall observed incidence rate 29–56 d		
No. of treatment periods at Day 29	1,596	2,284
Cases of ILD/person-weeks	11/5,797	15/8,392
Incidence rate per week (95% CI)	0.00190 (0.00078–0.00302)	0.00179 (0.00088–0.00269)
Overall observed incidence rate 57–84 d		
No. of treatment periods at Day 57	1,328	1,890
Cases of ILD/person-weeks	12/4,911	7/6,930
Incidence rate per week (95% CI)	0.00244 (0.00106–0.00383)	0.00101 (0.00026–0.00176)
Naive cumulative incidence after 84 d (first treatment periods only)		
Cases of ILD/no. of patients	59/1,482	35/1,677
Cumulative incidence (95% CI)	3.98% (3.04–5.11%)	2.09% (1.46–2.89%)
Kaplan-Meier cumulative incidence after 84 d (first treatment periods only)		
Cases of ILD/no. of patients	59/1,482	35/1,677
Cumulative incidence (95% CI)	4.50% (3.37–5.64%)	2.40% (1.61–3.20%)

Definition of abbreviations: ILD = interstitial lung disease; CI = confidence interval.

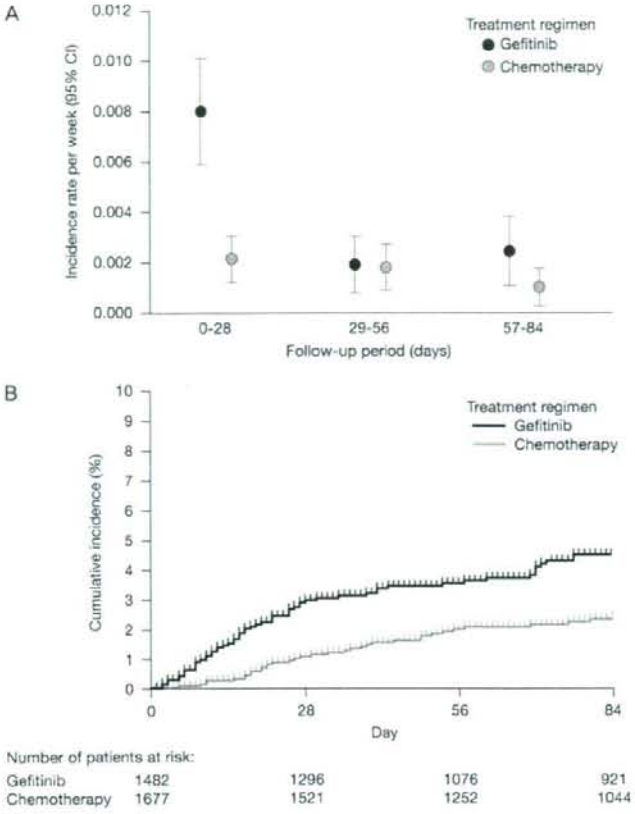


Figure 1. (*A*) Incidence rates of acute interstitial lung disease (ILD) in Japanese patients with non–small cell lung cancer for gefitinib and chemotherapy cohorts by 4-week period after treatment initiation. (*B*) Kaplan-Meier curves of risk of ILD to 12 weeks for the observed cohorts. CI = confidence interval.

study subjects, were also investigated as secondary and exploratory objectives in this study. These analyses are ongoing and results will be submitted for publication in due course.

Over the whole study follow-up, the average incidence rate for acute ILD events in patients treated with gefitinib was 3.2-fold higher relative to that seen with other chemotherapy treatments, adjusted for imbalances in other risk factors between treatments. The increased risk of ILD associated with gefitinib treatment was seen most clearly in the first 4 weeks after treatment initiation. Thus, increased physician awareness of risk factors and careful surveillance of Japanese patients during this period are indicated to manage risk. Such an approach is in line with current recommendations in Japan (20, 21). Beyond 4 weeks after treatment initiation, the risk of ILD associated with gefitinib treatment appears to fall.

ILD risk factors were found to be the same for both types of NSCLC therapy. Gefitinib is, however, a molecularly targeted agent. There is a significant body of evidence to indicate that gefitinib is a valid treatment option for some patients with NSCLC. In the IRESSA Survival Evaluation in Lung cancer (ISEL) study, a large phase III, placebo-controlled trial (n = 1,692), gefitinib was associated with some improvement in overall survival versus placebo, although this failed to reach statistical significance in the primary analysis of the overall population (22). Preplanned subgroup analyses from the study showed statistically significant differences in survival in favor of gefitinib in patients of Asian origin and those who had never smoked. Furthermore, tumor biomarker data suggest that patients with a high EGFR gene copy number, or an EGFR mutation, may be more likely to benefit (23, 24).

Therefore, the consideration of those patients more likely to benefit from the drug balanced with the better identification of these risk factors associated with ILD enables the physician to make careful judgment of the most appropriate therapy for the individual patient. Patients with several risk factors will generally be at more risk, and patients with risk factors may be at higher risk if gefitinib is used. This approach is facilitated by the fact that evidence to date suggests that subgroups less at risk of ILD tend to be those that respond well to gefitinib treatment (8).

A fatal outcome is the major concern with ILD as an SAE of drug treatment. In other large studies, fatality rates due to ILD in gefitinib-treated subjects of approximately 30% have been seen (8, 25), and a similar mortality was observed in this study in both gefitinib-treated and chemotherapy-treated ILD cases. The main predictors of a fatal outcome were older age (≥65 yr), smoking history, and preexisting ILD, as well as CT scan evidence of reduced normal lung (≤50%) or extensive areas adherent to pleura (≥50%). Because mortality is high among patients with NSCLC and the frequency of ILD in Japanese patients with NSCLC is low in comparison, ILD-related mortality impacted the overall survival at 12 weeks, for the cohort of

TABLE 5. RISK FACTORS FOR ACUTE ILD IDENTIFIED IN THE STUDY AND ESTIMATED ODDS RATIOS

Risk Factors	Odds Ratio (95% CI)
Treatment: gefitinib vs. chemotherapy	3.23 (1.94–5.40)
Age: ≥55 vs. ≤54 yr	1.92 (0.91–4.09)
WHO performance status	
1 vs. 0	1.57 (0.83–2.97)
2–3 vs. 0	4.02 (1.85–8.74)
Duration of NSCLC	
0.5 to <1 vs. <0.5 yr	0.65 (0.37–1.14)
≥1 vs. <0.5 yr	0.35 (0.20–0.62)
Concurrent cardiac disease: yes vs. no	2.44 (0.88–6.80)
Severity of preexisting pulmonary emphysema	
Mild vs. no	1.57 (0.89–2.79)
Moderate vs. no	1.04 (0.49–2.23)
Severe vs. no	0.47 (0.16–1.40)
Never-smoker and high extent of normal lung on CT (60–100%) (reference)	1.00 (reference)
Never-smoker and reduced extent of normal lung on CT (10–50%)	7.22 (2.52–20.64)
Smoker and high extent of normal lung on CT (60-100%)	4.43 (1.87–10.47)
Smoker and reduced extent of normal lung on CT (10–50%)	5.42 (2.08–14.12)
No preexisting ILD and high extent of normal lung on CT (60–100%) (reference)	1.00 (reference)
No preexisting ILD and reduced extent of normal lung on CT (10–50%)	7.22 (2.52–20.64)
Mild preexisting ILD and high extent of normal lung on CT (60–100%)	4.80 (1.83–12.63)
Mild preexisting ILD and reduced extent of normal lung on CT (10–50%)	6.08 (1.09–33.98)
Moderate–severe preexisting ILD and high extent of normal lung on CT (60–100%)	5.55 (1.40–21.99)
Moderate–severe preexisting ILD and reduced extent of normal lung on CT (10–50%)	25.27 (5.74–111.28)

Definition of abbreviations: CI = confidence interval; CT = computed tomography; ILD = interstitial lung disease; NSCLC = non–small cell lung cancer; WHO = World Health Organization.

gefitinib-treated patients, only to a limited extent (85.4 to 84%). Accordingly, there needs to be an appropriate individualized risk–benefit evaluation for patients also considering other treatments, many of which have their own problems with treatment-related mortality due to SAEs other than ILD.

Some methodologic issues may have influenced the study results and deserve comment. This kind of observational pharmacoepidemiologic study is generally considered sensitive to confounding by indication. Most often, it is assumed that more "sick" or "susceptible" patients will receive a new treatment,

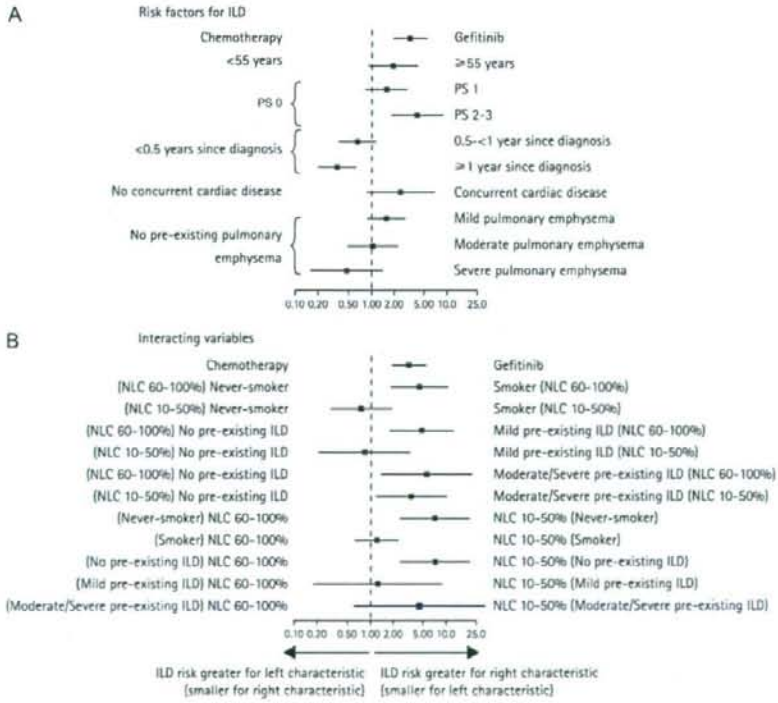
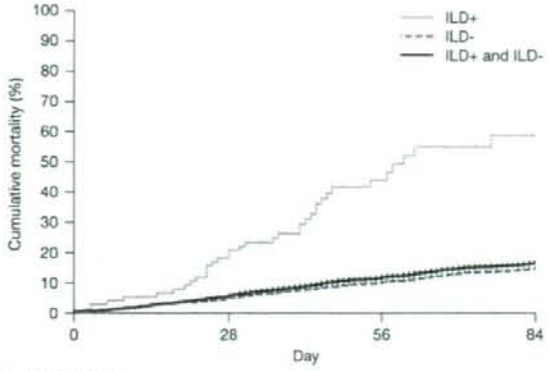


Figure 2. Adjusted odds ratios for risk factors for acute interstitial lung disease (ILD) in Japanese patients with non–small cell lung cancer from final logistic model. NLC = normal lung coverage (extent of normal lung on computed tomography scan); PS = World Health Organization performance status.



Number of patients at risk:

ILD+	78	64	22	11
ILD-	1771	1694	1416	1054
ILD+ and ILD-	1849	1758	1438	1065

Figure 3. Kaplan-Meier curves showing risk of death to 12 weeks in the gefitinib cohort overall and subdivided into those that developed interstitial lung disease (ILD+) and those who did not (ILD−).

leading to possibly more adverse effects in this group, even in the absence of a true relationship to treatment. Attempts to adjust for confounding using collected data would then push the adjusted estimate of effect closer to the null, but if sufficiently precise information on strong confounders cannot be collected, it may be impossible to remove all of the confounding. In conducting this study, the suspected adverse effect of ILD was recognized, and in the clinical setting, recommendations were in place to proceed with caution when treating some patients with suspected elevated baseline risk of ILD. This kind of selection would tend to produce the type of data pattern that was in fact observed in this study, a pattern of negative confounding that produces a more elevated OR when adjustment for confounders is performed. Thus, the results are well in line with what might be expected.

Misdiagnosis of ILD (outcome misclassification) is another concern, but it is expected that the stringent design features have minimized this problem in the present study (*see* online supplement for details). The diagnostic CRB review is a key feature, but it was still CT based, and biopsies—generally considered the gold standard for ILD diagnosis—were in most cases not taken. Overall, a sensitivity analysis suggested that, under reasonable assumptions about possible misclassification of ILD, the main result would remain similar and the conclusions from the study would not be greatly changed.

Random error is another consideration. However, although random error may be responsible for some bias in the point estimate, the confidence interval is reasonably narrow. The results are also consistent with other recent data. For example, as of January 2006, the estimated reporting rate of ILD-type events in Japan from the AstraZeneca Global Drug Safety Database of patients receiving gefitinib treatment was approximately 3.1% (26); from a retrospective study by the West Japan Thoracic Oncology Group (WJTOG), which studied 1,719 patients receiving gefitinib of whom 69 developed ILD, the frequency was 3.5% (95% CI, 2.8–4.5%) (8); from a postmarketing surveillance (PMS) study conducted by AstraZeneca KK Japan, which included 3,322 gefitinib-treated patients, it was 5.8% (25); whereas from the present study, the cumulative incidence at 12 weeks was 4.0% (95% CI, 3.0–5.1%).

These estimates are quite similar, even recognizing that the populations and selection of patients differ between these samples, and duration of follow-up, although similar, varies. In the present study, for the first time, an estimate of cumulative incidence of ILD after 12 weeks of treatment was obtained also from a chemotherapy-treated patient group; this frequency was 2.1% (95% CI, 1.5–2.9%), providing an estimate of this problem unrelated to gefitinib in patients with NSCLC in Japan.

The prognosis for gefitinib-treated patients who were diagnosed with ILD was also quite consistent with other studies. In the PMS study, ILD-related death among patients diagnosed with ILD was 38.6% (25); in the WJTOG study it was 44.3% (8); in the AstraZeneca Global Drug Safety Database as of January 2006, the proportion of ILD-type events with a fatal outcome in patients receiving treatment with gefitinib in Japan was 37.3% (AstraZeneca, data on file); and in the present study it was 31.6%. This proportion was quite similar to the chemotherapy-treated group, 27.9% (adjusted OR, 1.05; 95% CI, 0.4–3.2).

The factors associated with risk of acute ILD observed in this Japanese NSCLC population are largely different or even complementary to factors that predict better response to gefitinib. This would seem to support a hypothesis that the mechanism by which ILD occurs is distinct from the successful cancer response mechanism, offering a potential path toward selecting patients with optimal risk–benefit balance for gefitinib treatment.

Interestingly, the issue of ILD in patients with NSCLC, after gefitinib or other treatments, appears to be a problem largely limited to Japan. From the AstraZeneca Global Drug Safety Database, the reporting rate of ILD-type events in patients receiving treatment with gefitinib was only 0.23% in the rest of the world excluding Japan, based on more than 215,000 patients worldwide estimated to have been exposed to gefitinib (26). Even for neighboring countries, the pattern differs from Japan: the rate for East Asian countries, including Korea and Taiwan but excluding Japan, was 0.17% (26). The proportion of ILD-type events with a fatal outcome was similar, however: 37% in Japan and 31% in the rest of the world. The reasons for this difference in incidence of ILD between Japan and other countries remain unclear, but may relate to both constitutional and environmental factors specific to Japan or Japanese patients. For other drug treatments, too, a higher incidence of ILD has been noted in Japan than elsewhere (12, 13).

Within the study, some exploratory analyses are still ongoing related to genetic and proteomic predictors for ILD in patients with NSCLC, to search for biomarkers for early recognition of ILD and hopefully individualized risk assessment. This may

help to shed light on why ILD appears to be a particular issue for Japanese patients and the possible underlying mechanisms.

The EGFR is expressed on a number of constituent cells of the lungs including epithelium, smooth muscle cells, fibroblasts, and endothelium (27). There have been a number of animal studies using bleomycin- and vanadium pentoxide–induced lung injury with EGFR–tyrosine kinase inhibitors to determine the role of EGFR in lung fibrosis. Gefitinib and AG1478 have been used in such studies of mice and, when administered in a range of therapeutic doses, show clear attenuation of both bleomycin- (28) and vanadium pentoxide–induced (29) lung fibrosis, although one study (30) has shown augmentation of bleomycin-induced fibrosis (when using a subtoxic dose of gefitinib). The similarity of study design and choice of animal strain in the bleomycin studies make it difficult to explain the discrepant results other than by the excessive dosing. This leaves uncertainty as to the underlying mechanism of lung fibrosis in patients with NSCLC receiving gefitinib.

In summary, the study appears to be of adequate validity to avoid serious systematic biases, random error does not seem to be the most likely explanation for the results, and the observed increased risk of ILD with gefitinib treatment relative to chemotherapy treatment in Japanese patients is consistent with previous studies. Although preexisting ILD was confirmed as an important determinant of developing acute ILD symptoms after treatment with gefitinib or chemotherapy, the results also suggested that risk of ILD may be generally affected by a variety of other factors that decrease the amount of normally functioning lung tissue or affect the capability of tissue repair and recovery. The study thus identified several risk factors apart from treatment, which included preexisting ILD, which were not treatment specific, and which were partly similar to risk factors for idiopathic or rheumatic pulmonary lung fibrosis. These findings taken together suggest that there may be a common etiology that gives some patients a greater susceptibility both to idiopathic or rheumatic pulmonary fibrosis and to acute drug-induced lung injury after various treatments.

Conflict of Interest Statement: S.K. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. H.K. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. Y.N. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. M.F. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. K.N. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. Y.I. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. M.T. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. S.Y. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. K.N. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. M.S. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. The Japan Thoracic Radiology Group does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. H.J. has been an AstraZeneca employee since 2001. Y.I. has been an AstraZeneca employee since 2000. A.A. is a full-time employee of AstraZeneca. C.W. has been a full-time employee at AstraZeneca since 2001 until present and owns shares in the company. T.H. is a full-time R&D scientist at AstraZeneca, UK, and received stock options. F.N. is a full-time employee of AstraZeneca and owns shares in the company.

Acknowledgment: The authors thank the study monitors, nurses, data managers, other support staff, and the patients participating in the study; the external epidemiology advisory board (Kenneth J. Rothman, Jonathan M. Samet, Toshiro Takezaki, Kotaro Ozasa, Masahiko Ando) for their advice and scientific review of study design, conduct, and analysis; Professor Nestor Müller for his expert input into radiologic aspects of ILD diagnosis; all case review board members (including Japan Thoracic Radiology Group members [with *]) individually (Moritaka Suga, Takeshi Johkoh*, Masashi Takahashi*, Yoshiharu Ohno*, Sonoko Nagai, Yoshio Taguchi, Yoshikazu Inoue, Takashi Yana, Masahiko Kusumoto*, Hiroaki Arakawa*, Akinobu Yoshimura, Makoto Nishio, Yuichiro Ohe, Kunihiko Yoshimura, Hiroki Takahashi, Yukihiko Sugiyama, Masahito Ebina, and Fumikazu Sakai*) for their valuable work in blindly reviewing ILD diagnoses, as well as prestudy CT scans for preexisting comorbidities; and all hospitals and clinical investigators who contributed to the data collection in the study (*see below*). The authors thank Sarah Charlesworth, from Complete Medical Communications, who provided editing assistance funded by AstraZeneca.

Hospitals and principal investigators contributing to the study: National Hospital Organization Hokkaido Cancer Centre (Hiroshi Isobe), Hokkaido University Hospital (Koichi Yamazaki), National Hospital Organization Dohoku National Hospital (Yuka Fujita), Tohoku University Hospital (Akira Inoue), Sendai Kousei Hospital (Shunichi Sugawara), National Cancer Centre Hospital East (Yutaka Nishiwaki), Nippon Medical School Chiba Hokusoh Hospital (Yasushi Ono), Tokyo Medical University Hospital (Masahiro Tsuboi), Nippon Medical School Hospital (Tetsuya Okano), Toho University Omori Medical Centre (Nobuyuki Hamanaka), Toranomon Hospital (Kunihiko Yoshimura), National Hospital Organization Tokyo Hospital (Atsuhisa Tamura), Juntendo University Hospital (Kazuhisa Takahashi), Kyorin University Hospital (Tomoyuki Goya), Tokai University Hospital (Kenji Eguchi), Kitasato University School of Medicine (Noriyuki Masuda), Kanagawa Cardiovascular and Respiratory Centre (Takashi Ogura), Niigata Cancer Centre Hospital (Akira Yokoyama), National Nishi-Niigata Central Hospital (Hiromi Miyao), Toyama University Hospital (Muneharu Maruyama), Kanazawa University Hospital (Kazuo Kasahara), Aichi Hospital, Aichi Cancer Centre (Hiroshi Saito), National Hospital Organization Nagoya Medical Centre (Hideo Saka), Fujita Health University Hospital (Hiroki Sakakibara), Nagoya Ekisaikai Hospital (Masashi Yamamoto), Shiga University of Medical Science Hospital (Noriaki Tezuka), Kyoto Katsura Hospital (Takeshi Hanawa), National Hospital Organization Kyoto Medical Centre (Yoshiyuki Sasaki), Rinku General Medical Centre Municipal Izumisano Hospital (Hisao Uejima), Kinki University, School of Medicine (Kazuhiko Nakagawa), National Hospital Organization Kinki-chuo Chest Medical Centre (Masaaki Kawahara), Osaka City General Hospital (Koji Takeda), Osaka City General Hospital (Hirohito Tada), Osaka City University Hospital (Shinzoh Kudoh), Osaka Prefectural Medical Centre for Respiratory and Allergic Diseases (Kaoru Matsui), Osaka Police Hospital (Kiyoshi Komuta), Toneyama National Hospital (Soichiro Yokota), Kobe City General Hospital (Keisuke Tomii), Hyogo Medical Centre for Adults (Shunichi Negoro), Kobe University Hospital (Yoshihiro Nishimura), Institute of Biomedical Research and Innovation (Nobuyuki Katakami), Tenri Hospital (Yoshio Taguchi), Okayama University Medical and Dental School Hospital (Katsuyuki Kiura), Hiroshima City Hospital (Hidetaka Sumiyoshi), Hiroshima City Hospital (Noritomo Senoo), National Hospital Organization Shikoku Cancer Centre (Tetsu Shinkai), National Hospital Organization Kyushu Cancer Centre (Yukito Ichinose), Fukuoka National Hospital (Akira Motohiro), University of Occupational and Environmental Health (Masamitsu Kido), University of Occupational and Environmental Health (Kenji Sugio), National Hospital Organization Nagasaki Medical Centre (Akitoshi Kinoshita), Kumamoto University Hospital (Mitsuhiro Matsumoto), Kumamoto-Chuo Hospital (Sunao Ushijima), Okinawa National Hospital (Mutsuo Kuba).

References

1. American Thoracic Society. American Thoracic Society/European Respiratory Society international multidisciplinary consensus classification of the idiopathic interstitial pneumonias. *Am J Respir Crit Care Med* 2002;165:277–304.
2. Inoue A, Saijo Y, Maemondo M, Gomi K, Tokue Y, Kimura Y, Ebina M, Kikuchi T, Moriya T, Nukiwa T. Severe acute interstitial pneumonia and gefitinib. *Lancet* 2003;361:137–139.
3. Kondoh Y, Taniguchi H, Kawabata Y, Yokoi T, Suzuki K, Takagi K. Acute exacerbation in idiopathic pulmonary fibrosis: analysis of clinical and pathologic findings in three cases. *Chest* 1993;103:1808–1812.
4. Wells AU, Hogaboam CM. Update in diffuse parenchymal lung disease 2006. *Am J Respir Crit Care Med* 2007;175:655–660.
5. Raghu G, Nyberg F, Morgan G. The epidemiology of interstitial lung disease and its association with lung cancer. *Br J Cancer* 2004;91:S3–S10.
6. Kudoh S, Takeda K, Nakagawa K, Takada M, Katakami N, Matsui K, Shinkai T, Sawa T, Goto I, Semba H, *et al.* Phase III study of docetaxel compared with vinorelbine in elderly patients with advanced non-small-cell lung cancer: results of the West Japan Thoracic Oncology Group Trial (WJTOG 9904). *J Clin Oncol* 2006;24:3657–3663.
7. Abid SH, Malhotra V, Perry MC. Radiation-induced and chemotherapy-induced pulmonary injury. *Curr Opin Oncol* 2001;13:242–248.
8. Ando M, Okamoto I, Yamamoto N, Takeda K, Tamura K, Seto T, Ariyoshi Y, Fukuoka M. Predictive factors for interstitial lung disease, antitumor response, and survival in non-small-cell lung cancer patients treated with gefitinib. *J Clin Oncol* 2006;24:2549–2556.
9. Danson S, Blackhall F, Hulse P, Ranson M. Interstitial lung disease in lung cancer: separating disease progression from treatment effects. *Drug Saf* 2005;28:103–113.
10. Rossi SE, Erasmus JJ, McAdams HP, Sporn TA, Goodman PC. Pulmonary drug toxicity: radiologic and pathologic manifestations. *Radiographics* 2000;20:1245–1259.
11. Sandler AB, Nemunaitis J, Denham C, von Pawel J, Cormier Y, Gatzemeier U, Mattson K, Manegold C, Palmer MC, Gregor A, *et al.* Phase III trial of gemcitabine plus cisplatin versus cisplatin alone in patients with locally

advanced or metastatic non-small-cell lung cancer. *J Clin Oncol* 2000;18: 122–130.
12. Azuma A, Kudoh S. High prevalence of drug-induced pneumonia in Japan. *Japan Medical Association Journal* 2007;50:405–411.
13. Koo L, Clark J, Quesenberry CP, Higenbottam T, Nyberg F, Wolf M, Steinberg M, Forsythe B. National differences in reporting "pneumonia" and "pneumonia interstitial": an analysis of the WHO drug monitoring database on 15 drugs in nine countries for seven pulmonary conditions. *Pharmacoepidemiol Drug Saf* 2005;14:775–787.
14. Nyberg F, Hada S, Rothman KJ; Iressa CCS Collaborator Group. IRESSA and interstitial lung disease (ILD) in Japan: lessons from a large nested case-control study to evaluate a safety issue [abstract]. *Pharmacoepidemiol Drug Saf* 2006;15:S287.
15. Kudoh S, Kato H, Nishiwaki Y, Fukuoka M, Nakata K, Suga M, Jiang H, Itoh Y, Higenbottam T, Nyberg F; Japan Thoracic Radiology Group. A cohort and nested case-control study to quantify the risk of interstitial lung disease (ILD) in Japanese patients with NSCLC treated with gefitinib or chemotherapy [abstract]. *Am J Respir Crit Care Med* 2007;175:A148.
16. Pearce N. What does the odds ratio estimate in a case-control study? *Int J Epidemiol* 1993;22:1189–1192.
17. Brookhart MA, Schneeweiss S, Rothman KJ, Glynn RJ, Avorn J, Sturmer T. Variable selection for propensity score models. *Am J Epidemiol* 2006;163:1149–1156.
18. Hotta K, Kiura K, Tabata M, Harita S, Gemba K, Yonei T, Bessho A, Maeda T, Moritaka T, Shibayama T, *et al.* Interstitial lung disease in Japanese patients with non-small cell lung cancer receiving gefitinib: an analysis of risk factors and treatment outcomes in Okayama Lung Cancer Study Group. *Cancer J* 2005;11:417–424.
19. Takano T, Ohe Y, Kusumoto M, Tateishi U, Yamamoto S, Nokihara H, Yamamoto N, Sekine I, Kunitoh H, Tamura T, *et al.* Risk factors for interstitial lung disease and predictive factors for tumor response in patients with advanced non-small cell lung cancer treated with gefitinib. *Lung Cancer* 2004;45:93–104.
20. AstraZeneca KK Japan. Japanese patient information for IRESSA, version 17 [in Japanese]. Osaka, Japan: AstraZeneca KK Japan; October 2006.
21. Japan Lung Cancer Society Committee on Preparation of Guideline for Use of Gefitinib. Guideline for use of gefitinib. Chiba, Japan: The Society; 2005. In Japanese.
22. Chang A, Parikh P, Thongprasert S, Tan E-H, Perng R-P, Ganzon D, Yang C-H, Tsao C-J, Watkins C, Botwood N, *et al.* Gefitinib (IRESSA) in patients of Asian origin with refractory advanced non-small cell lung cancer: subset analysis from the ISEL study. *J Thorac Oncol* 2006;1:847–855.
23. Hirsch FR, Varella-Garcia M, Bunn Jr PA, Franklin WA, Dziadziuszko R, Thatcher N, Chang A, Parikh P, Rodrigues Pereira J, Ciuleanu T, *et al.* Molecular predictors of outcome with gefitinib in a phase III placebo-controlled study in advanced non-small-cell lung cancer. *J Clin Oncol* 2006;24:5034–5042.
24. Pao W, Miller VA. Epidermal growth factor receptor mutations, small-molecule kinase inhibitors, and non-small-cell lung cancer: current knowledge and future directions. *J Clin Oncol* 2005;23:2556–2568.
25. Yoshida S. The results of gefitinib prospective investigation [in Japanese]. *Medicine and Drug Journal* 2005;41:772–789.
26. Armour A. Gefitinib in advanced non-small cell lung cancer: clinical experience in patients of Asian origin. *Asia Pac J Clin Oncol* 2007;3:66–78.
27. Modi S, Seidman AD. An update on epidermal growth factor receptor inhibitors. *Curr Oncol Rep* 2002;4:47–55.
28. Ishii Y, Fujimoto S, Fukuda T. Gefitinib prevents bleomycin-induced lung fibrosis in mice. *Am J Respir Crit Care Med* 2006;174:550–556.
29. Rice AB, Moomaw CR, Morgan DL, Bonner JC. Specific inhibitors of platelet-derived growth factor or epidermal growth factor receptor tyrosine kinase reduce pulmonary fibrosis in rats. *Am J Pathol* 1999; 155:213–221.
30. Suzuki H, Aoshiba K, Yokohori N, Nagai A. Epidermal growth factor receptor tyrosine kinase inhibition augments a murine model of pulmonary fibrosis. *Cancer Res* 2003;63:5054–5059.

Association of epidermal growth factor receptor (*EGFR*) gene mutations with *EGFR* amplification in advanced non-small cell lung cancer

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(Received May 28, 2008/Revised August 5, 2008/Accepted August 6, 2008/Online publication October 16, 2008)

Somatic mutations in the epidermal growth factor receptor (*EGFR*) gene are associated with the response to *EGFR* tyrosine kinase inhibitors in patients with non-small cell lung cancer (NSCLC). Increased *EGFR* copy number has also been associated with sensitivity to these drugs. However, given that it is often difficult to obtain sufficient amounts of tumor tissue for genetic analysis from patients with advanced NSCLC, the relationship between these two types of *EGFR* alterations has remained unclear. We have now evaluated *EGFR* mutation status both by direct sequencing and with a high-sensitivity assay, the Scorpion-amplification-refractory mutation system, and have determined *EGFR* copy number by fluorescence *in situ* hybridization (FISH) analysis in paired tumor specimens obtained from 100 consecutive patients with advanced NSCLC treated with chemotherapy. *EGFR* mutations or FISH positivity (*EGFR* amplification or high polysomy) were apparent in 18% (18/100) and 32% (32/100) of patients, respectively. The Scorpion-amplification-refractory mutation system was more sensitive than direct sequencing for the detection of *EGFR* mutations. Furthermore, *EGFR* mutations were associated with *EGFR* amplification ($P = 0.009$) but not with FISH positivity ($P = 0.266$). Our results therefore suggest the existence of a significant association between *EGFR* mutation and *EGFR* amplification in patients with advanced NSCLC. (*Cancer Sci* 2008; 99: 2455–2460)

The epidermal growth factor receptor (EGFR) is a receptor tyrosine kinase of the ErbB family and has been implicated in the proliferation and survival of cancer cells. Aberrant expression of *EGFR* has been detected in many human epithelial malignancies, including non-small cell lung cancer (NSCLC).[1,2] This receptor has therefore been identified as a promising target for anticancer therapy, and several agents have been synthesized that inhibit its tyrosine kinase activity. *EGFR* tyrosine kinase inhibitors (TKI) have been evaluated most extensively in individuals with NSCLC, and they have had a substantial impact on the treatment of this disease by offering additional therapeutic options for patients with advanced NSCLC.[3–6]

Somatic mutations in the tyrosine kinase domain of *EGFR* have been detected in a subset of NSCLC patients who respond to EGFR TKI[7–9] and have been shown to be closely associated with sensitivity to these drugs.[10–14] Indeed, we and others have prospectively demonstrated a high response rate to EGFR TKI therapy in NSCLC patients with *EGFR* mutations.[15–21] An increased copy number of the *EGFR* gene, as revealed by fluorescence *in situ* hybridization (FISH), has also emerged as an effective molecular marker of EGFR TKI sensitivity in NSCLC.[22–24] We previously showed that *EGFR* mutation and *EGFR* amplification are associated in human NSCLC cell lines and that endogenous EGFR expressed in such cell lines positive for both of these *EGFR* alterations are activated constitutively.[25] However, the relationship between *EGFR* mutation and FISH positivity for *EGFR*, which reflects gene amplification or high polysomy, has remained unclear.[22–24,26,27] Indeed, only a few studies have evaluated the relationship between mutation and gene copy number for *EGFR* because of the difficulty in obtaining tumor samples suitable for genetic analysis from individuals with advanced NSCLC. We previously showed that the Scorpion-amplification-refractory mutation system (ARMS) is a sensitive technique for the detection of *EGFR* mutations in tumor specimens such as pleural effusion fluid or tissue obtained by transbronchial needle aspiration.[28–30] In the present study, we evaluated *EGFR* mutation status in small tumor specimens from patients with advanced NSCLC both by direct sequencing and by Scorpion-ARMS and compared the sensitivity of these methods for the detection of *EGFR* mutations. Furthermore, we determined *EGFR* copy number by FISH analysis in paired tumor specimens and examined its relationship to *EGFR* mutation.

Materials and Methods

Patients. The present retrospective study recruited consecutive patients with advanced NSCLC who received chemotherapy at Kinki University Hospital between January 2003 and December 2005. Patients eligible for the study had histologically confirmed stage III or IV NSCLC that was not curable by surgical resection or radiotherapy, irrespective of the presence of measurable lesions or good performance status (PS). Patients with recurrence after surgical resection were excluded. Complete clinical information and tissue blocks suitable for genetic analysis were available for 100 patients. We examined the relationship between *EGFR* mutation and *EGFR* copy number as well as the influence of these *EGFR* alterations on clinical outcome. Tumor response was assessed by computed tomography and evaluated according to the Response Evaluation Criteria in Solid Tumors.[31] Survival was calculated from the date of initiation of chemotherapy either to the date of death from any cause or to the date of last contact. Some patients had been receiving EGFR TKI treatment before the demonstration in 2004 that mutations in *EGFR* confer increased sensitivity to these drugs. Moreover, many patients had already died before the initiation of our genetic analysis, preventing us from obtaining informed consent. The institutional review board

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doi: 10.1111/j.1349-7006.2008.00962.x

therefore approved our study protocol with the conditions that samples would be processed anonymously and analyzed only for somatic mutations (not for germline mutations) and that the study would be disclosed publicly, according to the Ethical Guidelines for Human Genome Research published by the Ministry of Education, Culture, Sports, Science, and Technology, the Ministry of Health, Labor, and Welfare, and the Ministry of Economy, Trade, and Industry of Japan. The present study also conforms to the provisions of the Declaration of Helsinki.

Identification of *EGFR* mutations. The tumor specimens were fixed with formalin and embedded in paraffin. DNA was extracted with the use of a QIAamp Micro kit (Qiagen K.K., Tokyo, Japan) from tumor tissue derived either by macrodissection or by laser-capture microdissection carried out to enrich tumor cells. Polymerase chain reaction-based direct sequencing of exons 18–21 and ARMS with designed 'Scorpion' primers were applied for the allele-specific detection of *EGFR* mutations. Only the following previously described mutations[7,8] were classified as mutations in the present study: G719X in exon 18, deletion of E746 to A750 or of neighboring residues in exon 19, as well as L858R and L861Q in exon 21. Patients were regarded as *EGFR* mutation positive if a mutation in *EGFR* was detected either by direct sequencing or by ARMS. All mutations were confirmed by analysis of at least two independent amplification products.

Determination of *EGFR* copy number. *EGFR* copy number was determined by FISH analysis with the use of dual-color DNA probes (LSI *EGFR* SpectrumOrange/CEP 7 SpectrumGreen; Vysis, Downers Grove, IL, USA). The tumor specimens were classified into six categories on the basis of the FISH results, as described previously.[22] Those with high polysomy (≥4 copies of *EGFR* in ≥40% of cells) or gene amplification (presence of a tight *EGFR* gene cluster and a ratio of *EGFR* to chromosome 7 of ≥2 or ≥15 copies of *EGFR* per cell in ≥10% of cells analyzed) were considered FISH positive, with those in the remaining categories being considered FISH negative.

Statistical analysis. The relationships among *EGFR* status, clinical characteristics, and tumor response to EGFR TKI were analyzed with Fisher's exact test as appropriate. Survival curves were constructed by the Kaplan–Meier method, and the differences in survival between patient subgroups were compared by the log-rank test. The impact of various factors on survival was evaluated by univariate and multivariate analysis according to the Cox regression model. A P-value <0.05 was considered statistically significant. All statistical analysis was carried out with StatView software (SAS Institute, Cary, NC, USA).

Results

Patient characteristics. Between January 2003 and December 2005, a total of 125 consecutive patients diagnosed histologically with advanced NSCLC underwent chemotherapy at Kinki University Hospital. Tissue specimens from 100 patients were assessable for both *EGFR* mutation and *EGFR* copy number. Of these specimens, 72 were obtained by bronchoscopic biopsy, 15 by percutaneous needle biopsy (12 from lung, two from bone, and one from lymph node), six by thoracoscopic biopsy, and seven by surgery for diagnosis or palliative therapy. The clinical characteristics of these 100 patients are shown in Table 1. Most of the patients were male (64%) and had a history of smoking (67%), and adenocarcinoma was the most prevalent tumor histology (61%). Most patients (83%) also had a good Eastern Cooperative Oncology Group PS (0 or 1), and 63% received second-line or subsequent rounds of chemotherapy. Fifty-three patients (53%) were treated with EGFR TKI. Seventy patients (70%) had died by the time of genetic analysis, with the median follow-up time for the 30 survivors being 14.6 months.

***EGFR* alterations in non-small cell lung cancer.** Patients were analyzed for *EGFR* mutations by direct sequencing of exons 18 through 21 and by Scorpion-ARMS (Table 2). *EGFR* mutations, consisting of in-frame deletions in exon 19 ($n = 5$) and point mutations in exon 21 ($n = 13$), were detected in 18 patients (18%). Eight *EGFR* mutations were detected by direct sequencing and 16 mutations were detected by Scorpion-ARMS. Ten of the 16 mutations detected by Scorpion-ARMS were not identified by direct sequencing. However, two of the deletions in exon 19 (E746_S752 and E746_T751) that were detected by direct sequencing were not identified by Scorpion-ARMS, given that the Scorpion primers were designed only for detection of the E746_A750 deletion in exon 19. *EGFR* mutations were significantly more frequent in tumors of women than in those of men (33 vs 9%), in adenocarcinomas than in tumors with other histologies (28 vs 3%), and in never-smokers than in smokers (42 vs 6%) (Fig. 1a). One of the 18 *EGFR* mutations was detected in a squamous cell carcinoma. Determination of *EGFR* copy number by FISH analysis revealed gene amplification in six patients and high polysomy in 26 patients, with 32 patients thus being classified as FISH positive (Table 3). In contrast to *EGFR* mutation, FISH

Table 1. Characteristics of patients with advanced non-small cell lung cancer ($n = 100$)

Characteristic	Subset	No. patients
Sex	Male	64
	Female	36
Smoking history	Never-smoker	33
	Smoker	67
Tumor histology	Adenocarcinoma	61
	Other	39
Eastern Cooperative Oncology Group performance status	0	24
	1	59
	≥2	17
No. chemotherapies	1	37
	≥2	63

Table 2. Detection of epidermal growth factor receptor (*EGFR*) mutations by direct sequencing or amplification-refractory mutation system (ARMS) ($n = 100$)

Site	Mutation	Direct sequencing	ARMS	Direct sequencing or ARMS
Exon 19	15-bp deletion	1	3	3
	16-bp deletion	1	0	1
	19-bp deletion	1	0	1
Exon 21	L858R	5	13	13
Total		8 (8%)	16 (16%)	18 (18%)

Table 3. Determination of epidermal growth factor receptor gene copy number by fluorescence *in situ* hybridization (FISH) analysis ($n = 100$)

FISH status	Finding	No. patients
Positive	Gene amplification	6
	High polysomy	26
	Total	32
Negative	Low polysomy	35
	High trisomy	2
	Low trisomy	26
	Disomy	5
	Total	68

doi: 10.1111/j.1349-7006.2008.00962.x