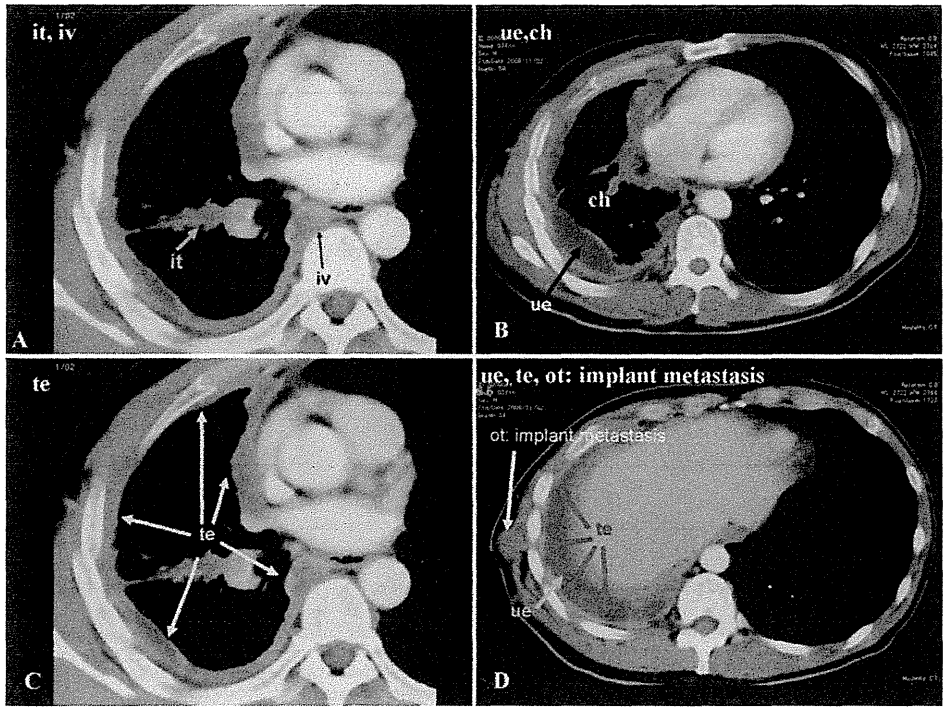

Fig. 4. A 45-year-old man with diffuse epithelioid MPM. CT scan shows interlobar fissure pleural thickening (A), invasion to the tissue near to the vertebral column ("iv") (black arrow) (A). There is unilateral pleural effusion ("ue") (black arrow) on the right hemithorax, leading to the contracted hemithorax ("ch") (B). Tumoral encasement of lung ("te") involvement on the right hemithorax (C, D) and an implant metastasis lesion are observed on the chest wall of right lung (D). According the CT appearance, MPM probability was agreed by experts as high probability Grade 4 at advanced severity. This is a typical MPM case.

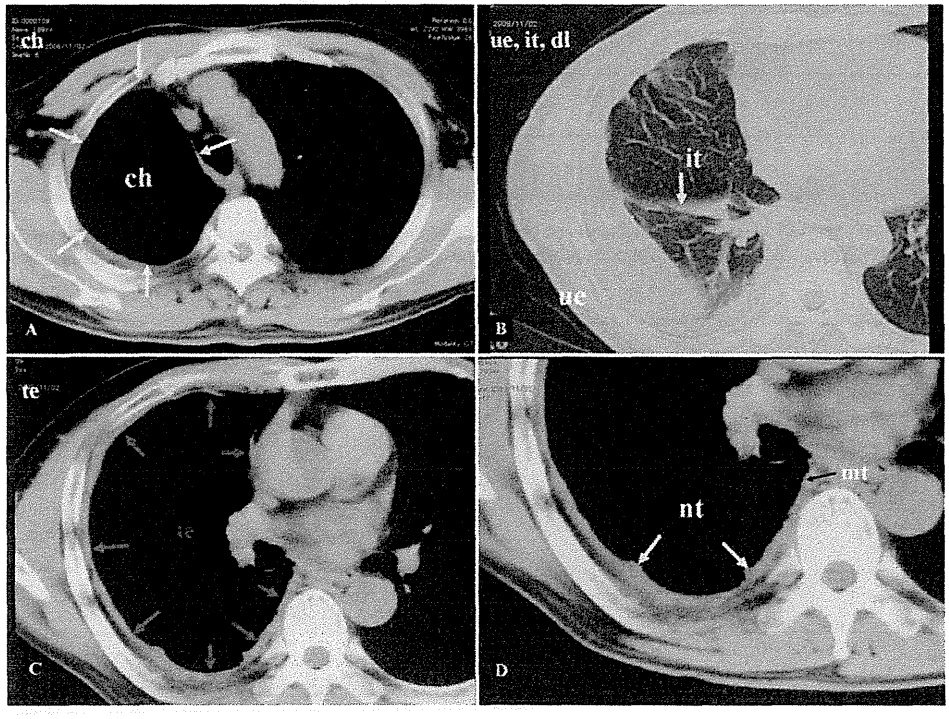

Fig. 5. A 58 year-old man with diffuse desmoplastic MPM. CT scan shows contracted hemithorax ("ch") leading to the right hemithorax volume loss (A). There is interlobar fissure pleural thickening ("it"), together with unilateral pleural thickening ("ue"), causing the lung volume diminished ("dl") (B). The involvement of tumoral encasement of lung ("te") (C), nodular pleural thickening ("nt") and mediastinal pleural thickening ("mt") (black arrow) on the right hemithorax are observed (D). MPM probability was agreed by experts as high MPM probability Grade 4 at advanced severity. This is a typical MPM case.

Table 2
Agreement on dichotomized MPM probability with 4 experts' consensus and sensitivity and specificity for MPM by three radiologists at the two trials.

Reader	Reading trial	Agreement with experts on dichotomized MPM probability	Sensitivity for MPM	Specificity for MPM
Radiologist 1	1st	18/22(81.82%)	16/20 (80%)	2/2 (100%)
	2nd	21/22(95.45%)	20/20 (100%)	1/2 (50%)
Radiologist 2	1st	19/22(86.36%)	17/20 (85%)	2/2 (100%)
	2nd	22/22 (100%)	20/20 (100%)	2/2 (100%)
Radiologist 3	1st	14/22(63.64%)	12/20 (60%)	2/2 (100%)
	2nd	18/22(81.82%)	16/20 (80%)	2/2 (100%)

Among the 20 definite MPM cases, the cases with MPM probability Grade 2, Grade 3 and Grade 4 accounted for 3/20 (15%), 7/20 (35%) and 10/20 (50%), respectively.

For the 3 cases with MPM probability Grade 2, the cases with the feature "nodular pleural thickening" ("nt") accounted for 1/3(33.33%), and the cases with "invasion" ("iv") accounted for 2/3 (66.67%).

Among the 7 cases with MPM probability Grade 3, the cases with feature "medialstinal pleural thickening" ("mt") accounted for 7/7 (100%), the feature of "unilateral pleural effusion" ("ue") were present in 5/7 (71.43%), "interlobar fissure thickening" ("it") in 4/7 (57.14%), "diminished lung" ("dl") in 4/7 (57.14%), "nt" in 2/7 (28.57%), and "iv" in 1/7 (14.29%).

Among the 10 cases with MPM probability Grade 4, the most frequently recorded features were "nt" in 10/10 (100%), "mt" in 100%, and "dl" in 100%. The cases with feature "ue" were in 80%, "it" in 70%, "iv" in 60%, and "tumoral encasement of lung" in 50%.

3.2. The sensitivity and specificity for MPM by three radiologists, the agreement on MPM probability by radiologists with experts

The sensitivity for MPM, specificity for MPM and observed agreement on the dichotomized MPM probability by 3 radiologists at two times of CT readings before and after studying the MPM-CT Guideline are shown as in Table 2.

The sensitivity for MPM by the three radiologists at the 2nd trial was 100%, 100% and 80%, which was higher than 80%, 85% and 60%, respectively at the 1st reading trial. The observed agreements on dichotomized MPM probability by radiologists were increased at the 2nd trial compared with those at the 1st trial.

The values of weighted kappa for the agreement of MPM probability on the 4-point scale by three radiologists with experts were increased at the 2nd trial, compared with those at the 1st trial, as shown in Table 3.

The weighted kappa values for the agreement with experts on 4-point scale MPM probability by three radiologists were 0.32, 0.51 and 0.37 at the 2nd trial vs 0.24, 0.48 and 0.29 at the 1st trial, respectively. The kappa value for inter-reader agreements on the dichotomized MPM probability between radiologists and experts at the 2nd reading trial were 0.65 (good), 1 (excellent) and 0.42 (moderate), respectively, which were significantly higher than 0.42 (moderate), 0.51 (moderate) and 0.21 (fair) at the 1st trial, respectively. The average kappa for the agreement on dichotomized MPM probability between radiologists and experts were 0.69 (good) at the 2nd trial vs 0.38 (fair) at the 1st trial, which seemed to show an upgrading in reading skill.

3.3. The agreement on MPM features between radiologists and experts

The results of the agreements for the recorded MPM features between the three radiologists and experts are shown as in Table 4.

For radiologist 1, the agreement with experts for 6 features "unilateral pleural effusion" ("ue"), "nodular pleural thickening" ("nt"), "interlobar fissure thickening"("it"), "mediastinal pleural thickening" ("mt"), "invasion" ("iv") and "diminished lung" ("dl") at the 2nd reading trial was better compared with those at the 1st trial. The agreement for feature "ue" and "nt" at the 2nd trial was good (kappa = 0.62 and 0.61, respectively). The agreement for feature "tumoral encasement of lung" ("te") at the 2nd trial was excellent to the same extent as that at the 1st trial (kappa = 0.86). The agreement with experts on 3 features ("mt", "iv" and "dl") was markedly increased to excellent (kappa > 0.8) at the 2nd trial.

For radiologist 2, the kappa values for the agreement with experts were increased for 5 features ("ue", "nt", "mt", "te" and "dl") at the 2nd trial in comparison with those at the 1st trial. The kappa values for the features "ue", "nt" and "te" showed good agreement with experts (kappa > 0.6), and "mt" and "dl" showed excellent agreement (kappa > 0.8). The kappa values for the feature "it" and "iv" was 0.55 and 0.51 at the 2nd trial, which were lower than 0.73 and 0.61 at the 1st trial, respectively.

For the radiologist 3, the kappa values for 5 features ("nt", "mt", "te", "iv" and "dl") were increased at the 2nd trial in comparison with the 1st trial. The feature "nt" and "te" shows good agreement with expert (kappa = 0.64 and 0.77, respectively). The kappa value for the feature "it" at the 2nd trial was equal to that one at the 1st trial. The kappa value for the feature "ue" (0.49) at the 2nd trial was lower than at the 1st trial (0.65).

The average kappa values of 7 MPM CT features at the 2nd trial by the three radiologists were significantly increased in comparison with the 1st trial, and the six features "ue", "nt", "mt", "te" and "dl" showed good agreement between radiologists and experts (kappa > 0.60).

Table 5 shows that the kappa values for the agreement on the 7 MPM CT features by the three radiologists with experts were significantly higher than those at the 1st trial.

Table 3
Agreement on 4-point scale MPM probability and dichotomized MPM probability between radiologist and experts in terms of weighted kappa.

Reader	4-scale MPM probability K_w (95% CI)		Dichotomized MPM probability K_w (95% CI)	
	1st trial	2nd trial	1st trial	2nd trial
Radiologist 1	0.24 (−0.06, 0.54)	0.32 (0.06, 0.58)	0.42 (−0.09, 0.93)	0.65 (−0.03, 1)
Radiologist 2	0.48 (0.19, 0.76)	0.51 (0.24, 0.78)	0.51 (−0.01, 1)	1
Radiologist 3	0.29 (0.04, 0.53)	0.37 (0.12, 0.62)	0.21 (−0.22, 0.65)	0.42 (−0.09, 0.93)
Weighted kappa Mean(SD)	0.34 (0.13)	0.40 (0.10)	0.38(0.15)	0.69(0.29)

Table 4
Agreement for the recorded MPM CT features between radiologists and experts by kappa statistics.

Reader	Reading trial	Experts						
		ue	nt	it	mt	te	iv	dl
Radiologist 1	1st	0.32	0.35	0.36	0.20	0.86	0.25	0.49
	2nd	0.62	0.61	0.55	0.88	0.86	0.81	0.80
Radiologist 2	1st	0.65	0.31	0.73	0.68	0.60	0.61	0.73
	2nd	0.72	0.70	0.55	1	0.77	0.51	0.90
Radiologist 3	1st	0.65	0.37	0.45	0.39	0.28	0.25	0.17
	2nd	0.49	0.64	0.45	0.47	0.77	0.49	0.57
All radiologists Mean (SD)	1st	0.54 (0.19)	0.34 (0.03)	0.51 (0.19)	0.42 (0.24)	0.58 (0.29)	0.37 (0.21)	0.46 (0.28)
All radiologists Mean(SD)	2nd	0.61 (0.12)	0.65 (0.05)	0.52 (0.06)	0.78 (0.28)	0.80 (0.05)	0.60 (0.18)	0.76 (0.17)

Note: 1st and 2nd indicates the 1st CT reading trial and the 2nd CT reading trial, SD = standard deviation of kappa values.

Table 5
Comparative analysis for the kappa values for the agreement with experts on the 7 MPM CT features by all three radiologists between the two reading trials by means of 2-Related-Samples Nonparametric Test (Wicoxon Signed Rank Test).

Kappa 2–Kappa 1*	Number of pairs	Mean rank	Sum of ranks	*P*
Negative ranks[a]	3	4.67	14	<0.01
Positive ranks[b]	16	11	176	
Ties[c]	2			
Total	21			

Note: *Kappa 2: kappa value for the agreement by three radiologists with expert on the 7 features recorded at the 2nd reading trial. Kappa 1: kappa value for the agreement by three radiologists with expert on the 7 features recorded at the 1st reading trial.

[a] Kappa 2 < Kappa 1.
[b] Kappa 2 > Kappa 1.
[c] Kappa 2 = Kappa 1.

4. Discussion

With the development of modern technology, CT scans have now become routine clinical practice for detecting pleural abnormalities in patients. The diagnosis of MPM is usually based on the combination of occupational history, physical and laboratory examination, radiology and the thoracic pathology. CT findings are important to provide the clue in the diagnosis of MPM before any invasive biopsy procedures take place. Diagnosis of MPM at the early stage may enable patients to obtain a better outcome with multiple modality therapy including extensive surgery, chemotherapy, and radiotherapy, which may offer increases in survival time and the life quality for MPM patients [7].

The MPM-CT Guideline was developed by the international experts' efforts [2]. The Guideline provided a standardized way for physicians to record CT findings on subject CT films with the assistance of the MPM reference CT films with typical MPM features. These allow the physicians to make appropriate judgments for the MPM probability, which is determined by the overall impression of the CT findings as a whole and comprehensive evaluation on CT findings being consistent or inconsistent with MPM features, the severity of the diseases, and the distribution of MPM involvement.

Among the 20 definite MPM cases in the current study, the mostly seen features in the number were "mediastinal pleural thickening" ("mt") (85%), and then were "diminished lung" ("dl") (70%), the "unilateral pleural effusion" ("ue") (65%), "nodular pleural thickening" ("nt") (65%) and "interlobar fissure thickening" ("it") (55%). The cases with "invasion" accounted for 45%. Most of the 7 features are suggestive of MPM to somewhat through quite extent and frequently common on the CT images of MPM [8].

The relationship between the MPM probability and the number of 7-MPM-CT features in each case was investigated. Among the 20 MPM cases, those cases with the high MPM probability (Grade 3, 4) were found to have more of 7-MPM-CT features. It was suggested that the more features observed on the CT, the higher MPM probability for the case determined, presenting with a positive correlation. The severity in association with the feature may be the second reason related with probability of MPM. When the features are at severe disease stages, the cases may have high MPM probability, even with only a few features of these.

The relatively high prevalence and frequency of "unilateral pleural effusion" in MPM is of major diagnostic importance [9]. At the early stage of MPM, irrespective of normal-appearing pleura, "unilateral pleural effusion" can be the only finding. The main mechanism of pleural fluid formation in malignancy is lymphatic obstruction. For the cases with pleural effusion and a history of asbestos exposure, MPM should be considered and further investigation should be conducted [10].

There were two features listed in the MPM-CT Guideline "contracted hemithorax" ("ch") and "pleural mass" ("pm") not included in the current comparative analysis, because these two features were later added at the 2nd workshop to the proposed MPM-CT Guideline. The two features are crucial and helpful to make diagnosis of MPM. Solitary masses may occur at the early stage, while multiple masses are more common at later stages [11]. MPM tend to spread along the pleural surface in a "sheet-like" fashion [12]. In CT images with feature "ch", the involved hemithorax is noticeably contracted from a comparison with that in the contralateral lung.

The current study showed that after studying the MPM-CT Guideline, the sensitivity for MPM by all three radiologists was

increased at the 2nd trial compared with those at the 1st trial. For the 1st radiologist, although the specificity for MPM at the 2nd trial was lower than at the 1st trial, the observed agreement for the MPM probability was increased to 95.45% at the 2nd trial vs 81.82% at the 1st trial.

Kappa is affected by prevalence of the finding under observation [13,14]. In the current study, the proportion of the non-MPM cases among total case was only in 10%, and many cases (50%) had MPM probability Grade 2 or Grade 3, which had great variances between readers. Therefore the weighted kappa on 4-scale MPM probability proved to be low. However, the analysis with the dichotomized MPM probability showed that two radiologists obtained good and excellent agreements, i.e., kappa = 0.65 and 1, respectively. The 3rd radiologist had achieved moderate agreement with kappa 0.42 on dichotomized MPM probability at the 2nd trial vs kappa 0.21 at the 1st trial.

The kappa statistics showed that good through to excellent agreements with experts by two of the three radiologists were obtained at the 2nd trial for 5 and 6 features, respectively. For the radiologist 3, there were 5 features including "nt", "mt", "tumoral encasement of lung" ("te"), "invasion" ("iv") and "dl" with increased agreements with experts at the 2nd trial compared with those at the 1st trial. Compared with those at the 1st trial, the average kappa values of 7 features by the three radiologists were increased at the 2nd trial, in which the five features, i.e., "ue", "nt", "mt", "te" and "dl", showed good agreement with experts. Wicoxon Signed Rank Test showed that the kappa values for the agreement on the 7 MPM CT features between the three radiologists and experts were significantly higher than those at the 1st trial. These revealed that the radiologists had made improvement in recognition of the MPM CT features at the 2nd trial compared with the 1st trial.

Compared to the other features in the 20 MPM cases, the agreement with experts in the feature "invasion" was relatively lower. At the 2nd trial, the kappa for "invasion" was 0.51 by the 2nd radiologist and 0.49 by the 3rd radiologist, respectively. This implied that the feature "invasion" was difficult to identify compared to the other MPM features. One reason may be its frequent coexistence with other features that the appearance of this feature may become less notable. The severity of the invasion to the lung structure may be the second reason. If it was less severe, "invasion" may not be easy to identify. This feature of "invasion" is quite suggestive of malignancy. This feature overlaps with metastasis of other carcinoma [15], while the other malignancy also has lung parenchymal involvement.

The average kappa for the agreements between radiologists and experts on feature "interlobar fissure thickening" was 0.51 and 0.52 at the 1st and 2nd trials, respectively, which were lower than those of other features. The reason may be ascribed to the fact that "interlobar fissure thickening" in some cases is so slight that it may easily be neglected. The feature "interlobar fissure thickening" reflects tumor growth along the interlobar spaces, and may represent one of the earliest significant MPM features, which is seen less frequently in other malignancies or in benign pleural diseases [16].

The CT findings in MPM are not pathognomonic because similar findings may be found in metastatic carcinoma. Nevertheless, they are characteristic. The MPM features can provide valuable information to make a diagnosis of MPM. By identifying the MPM features on CT images, clinicians can recognize MPM [17]. It will be helpful for occupational physicians during the occupational disease screening in health surveillance for workers.

One limitation of the study was that there were only two non-MPM cases with pleural plaque. This may affect the specificity for MPM. Despite the limitation, the results of the comparative study showed that all three radiologists had improved their sensitivity for MPM after studying the MPM-CT Guideline. Two of them had obtained either good or excellent agreements with experts in identifying most of features at the 2nd trial. These suggested that reliability of the Guideline and the MPM reference CT films to improve the reading proficiency of the radiologists may be validated.

5. Conclusions

The current study suggested that the three radiologists improved the proficiency in diagnosis for MPM by identifying of MPM CT features after studying the MPM-CT Guideline with reference MPM CT features. The MPM-CT Guideline and reference CT films may act as good tool to facilitate physicians in recognition of MPM features and contribute to early diagnosis of MPM in the health surveillance.

Conflict of interest statement

None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

Role of the funding source

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ejrad.2012.05.022.

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Allelotypes of lung adenocarcinomas featuring *ALK* fusion demonstrate fewer onco- and suppressor gene changes

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Abstract

Background: A subset of lung adenocarcinomas harboring an *EML4-ALK* fusion gene resulting in dominant oncogenic activity has emerged as a target for specific therapy. *EML4-ALK* fusion confers a characteristic histology and is detected more frequently in never or light smokers and younger patients.

Methods: To gain insights into etiology and carcinogenic mechanisms we conducted analyses to compare allelotypes of 35 *ALK* fusion-positive and 95 -negative tumours using single nucleotide polymorphism (SNP) arrays and especially designed software which enabled precise global genomic profiling.

Results: Overall aberration numbers (gains + losses) of chromosomal alterations were 8.42 and 9.56 in tumours with and without *ALK* fusion, respectively, the difference not being statistically significant, although patterns of gain and loss were distinct. Interestingly, among selected genomic regions, oncogene-related examples such as 1p34.3 (*MYCL1*), 7q11.2(*EGFR*), 7p21.1, 8q24.21(*MYC*), 16p13.3, 17q12(*ERBB2*) and 17q25.1 showed significantly less gain. Also, changes in tumour suppressor gene-related regions, such as 9p21.3 (*CDKN2A*) 9p23-24.1 (*PTPRD*), 13q14.2 (*RB1*), were significantly fewer in tumours with *ALK* fusion.

Conclusion: Global genomic comparison with SNP arrays showed tumours with *ALK* fusion to have fewer alterations in oncogenes and suppressor genes despite a similar overall aberration frequency, suggesting very strong oncogenic potency of *ALK* activation by gene fusion.

Keywords: Lung adenocarcinoma, *ALK* fusion, SNP array, Allelotype, Copy number

Background

The adenocarcinoma is the most common form of lung cancer worldwide, different subsets having specific genetic backgrounds of great importance for molecular-targeted therapy. For example, somatic mutations of the epidermal growth factor receptor (*EGFR*) are especially prevalent in adenocarcinomas among never smokers, females, and those with Asian ethnicity [1]. On the other hand, *KRAS* mutations are associated with the smoking habit [2] and the two tend to be mutually exclusive. Recently, Soda et al. found a novel fusion gene, *EML4-ALK*, arising from an inversion on the short arm of chromosome 2 in non-small cell lung carcinomas [3]. *ALK* fusion is a unique example of tyrosine kinase activation by structural chromosome rearrangement [4].

EML4-ALK fusion is a powerful driving molecular event by itself. The chimeric protein permits ligand-independent dimerization and constitutive activation of *ALK*, resulting in dominant oncogenic activity. Multiple fusion variants of *EML4-ALK* and notable clinicopathological characteristics of fusion positive tumours have been revealed [5-9]. Since the tyrosine kinase is involved and activated by gene fusion, this type of malignancy has emerged as a target for anti-tyrosine kinase therapy [4,10-12].

We have revealed that *ALK* fusion-positive tumours constituted a particular subset in lung adenocarcinomas in

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terms of clinical characteristics, histology and etiology, as well as molecular changes [7,8]. It is of great interest to assess global genomic alterations to provide deep insight into their genesis, especially considering these tumours arise in non- or light smokers. Single nucleotide polymorphism (SNP) microarray analysis enables precise high-throughput detection of genomic copy number alterations, gains and losses in the genome contributing to carcinogenesis [13] with gene expression varying consistently with DNA copy number changes [14,15]. We therefore conducted of the present genomic profiling of lung adenocarcinomas with and without *ALK* fusion.

Methods

Patient population and specimens

A series of 130 cases of lung adenocarcinomas, 35 with *EML4-ALK* or *KIF5B-ALK* fusion and 95 cases without, were enrolled in this study. From 1998 to 2008, 1,086 primary lung adenocarcinomas were surgically resected at Thoracic Surgery Division, the Cancer Institute Hospital, Japanese Foundation for Cancer Research (JFCR), Tokyo. All cases were screened as to ALK expression by immunohistochemistry using the iAEP method [6] and for positive cases subsequent RT-PCR and FISH analysis were performed, as previously described [5,6,16]. Among them, sufficient amounts and quality of fresh tumour material were available for 35 cases. Fusion gene variants are listed in Addtional file 1: Table S1. V3 constituted the largest proportion, 31% (11/35), having a breakpoint at exon 20 of *EML4*. A rare variant, *KIF5B-ALK* fusion, was detected in two cases. There was no correlation with fusion variant and pathological subtypes (data not shown). The 95 cases without *ALK* fusion were randomly selected from 730 surgically resected adenocarcinomas from 1995 to 2003 at the same hospital. Tissue specimens were snap-frozen in liquid nitrogen, typically within 20 minutes after resection, and stored at −80°C until use. Genomic DNA was extracted by standard proteinase K digestion and the phenol-chloroform method. To confirm if specimens used for analysis in this study contained a significant amount of tumour cells, typically 50% or more, a neighboring surface was examined histologically with frozen sections. This study was approved by the institutional review board of the JFCR.

Mutation analysis of EGFR, KRAS and TP53

For *EGFR* mutation analysis, exons 18 to 21 were amplified by PCR with specific oligo-primers. For point mutations in exon 18, PCR products were directly sequenced. Fragment analysis was performed for exons 19 and 20 deletions and insertion mutations. The presence of one point mutation in exon 21 was detected by genotyping analysis. To examine *TP53* mutations, direct sequencing from exons 5 to 10 was carried out. For *KRAS* mutation analysis, codons 12, 13 and 61 were examined by direct sequencing. Primers and detailed procedures were as described previously [17].

Histological diagnosis and clinical staging

Histological diagnosis was made on the basis of World Health Organization (WHO) classification [18] by experienced pathologists (N.M. and Y.I.). Pathological staging was based on the AJCC/UICC staging manual of lung cancer [19]. Differentiation grading of adenocarcinoma was determined essentially according to the Japan Lung Cancer Society criteria as illustrated previously [20]. Briefly, well-differentiated (w/d) tumors are composed chiefly of glands lined by, or of papilla covered by, one-layered tumor cells. Also, Adenocarcinoma in situ (AIS) is included in this category. Moderately differentiated (m/d) lesions comprise glands showing a cribriform pattern, fused with one another, or glands lined by, or papillae covered by, tumor cells demonstrating obvious piling-up. Poorly differentiated (p/d) carcinomas show mainly solid growth and only occasionally glandular/papillary patterns and/or mucus production. Blood vessel and lymphatic invasion was also explored microscopically, with hematoxylin-eosin and elastic-fiber stained sections of maximum tumour diameter made from paraffin-embedded specimens.

SNP array analysis and comparisons of allelic imbalance at the chromosome arm level and in selected cancer-related regions

Extracted DNA was subjected to Affymetrix GeneChip Mapping 250K arrays. Allelic imbalance was analyzed using software termed the Copy Number Analyzer for Affymetrix Gene Chip Mapping (CNAG Ver. 2.0) [21]. After appropriate normalization of mean array intensities, signal ratios between tumours and anonymous normal references were calculated in an allele-specific manner, and allele-specific copy numbers were inferred from the observed signal ratios based on the hidden Markov model using the CNAG/AsCNAR software [21-23]. With this procedure, genomic profiles of *ALK* fusion-positive and -negative tumours were obtained. Datas have been deposited at NCBI's Gene Expression Omnibus data repository under GEO series accession number GSE41536.

Comparison was at two levels; a chromosome arm level and a smaller, specific gene locus level. To do this, first we compared average numbers of chromosome arms altered between the two groups [24]. We called gain or loss of each chromosomal arm when copy number change stretched more than 80% of entire length. Secondly, we compared recurrent copy number aberrations at twenty-one cancer-related loci with gains and five with losses. These specific regions were selected based on previous studies of the lung cancer genome

[25,26] and through our global mapping with CNAG. The selected regions with relevant genes were as follows: for gains, 1p34.3 (*MYCL1*), 1q21.2 (*S100 family*), 3q29 (*MUC4*), 5p15.33 (*TERT*), 6p21.1 (*VEGF*), 7p11.2 (*EGFR*), 7p21.1, 7q31.2 (*MET*), 8q24.21 (*MYC*), 10q11.22, 12p12.1 (*KRAS*), 12q14.1 (*CDK4*), 12q15 (*MDM2*), 14q13.3 (*TTF1*), 16p13.3, 17q12 (*ERBB2*), 17q25.1, 19q12 (*CCNE1*), 20q13.2, 20q13.32, 20q13.33 (*TNFSF6B*); and for losses, 9p21.3 (*CDKN2A*), 9p23-p24.1 (*PTPRD*), 10q23.31 (*PTEN*), 13q14 (*RB1*), 17p13.1 (*TP53*).

Statistical analysis

Clinicopathological parameters of cases with or without *ALK* fusion and the frequencies of chromosome arms changed and copy numbers of targeted loci were compared by the chi-square test or the Fisher's exact test as appropriate. The average number of chromosome arms altered with or without *ALK* fusion was compared with Students' *t*-test. Statistical significance was defined as P=0.05 or less.

Results

Comparisons of clinicopathological profiles of tumours with or without *ALK* fusion

Clinicopathological profiles of patients are summarized in Table 1. *ALK* fusion-positive cases were significantly younger and featured significantly more never-smokers (P=0.05, P=0.004, respectively). *ALK* fusion-positive tumours were histologically adenocarcinomas with notable characteristics such as poor differentiation as well as an acinar type structure and mucin production, as reported previously [7-9]. In this study, distribution of histological subtypes differed between two groups, namely, "acinar" subtype accounted for nearly forty percent in *ALK* fusion positive group (Table 1). The frequencies of vascular invasion, both of blood and lymph vessels, did not significantly differ between the two groups (P=0.738, P=0.273, respectively). In addition, the distribution of pathological stages did not vary (P=0.532).

Mutational status of TP53, EGFR and KRAS

Data for the mutational status of *TP53, EGFR and KRAS* in the two groups are summarized in Table 1. Twenty-one cases had *TP53* mutations. Only one case with *ALK* fusion (Case 9: 1/35, 3%) harbored a mutation, a G/A transition at codon 273, as compared to 20 cases without *ALK* fusion (20/95, 21%), the mutation rates being significantly different (P=0.014) (Table 1, Additional file 1: Table S2). Twelve (12/21, 57%) of the *TP53*-mutated cases had a smoking history.

EGFR and *KRAS* mutations were not detected among *ALK* fusion-positive tumours. This fact that *ALK* rearrangement was mutually exclusive with *EGFR* and *KRAS* mutations (P<0.0001, P=0.189, respectively) is in line with our previous studies [8]. The *EGFR* mutation rate was 58% (55/95) in *ALK* fusion-negative cases and decreased with the smoking burden: 70.7% (29/41) in never smokers, 62.5% (15/24) in light smokers (0<pack-years<20) and 36.7% in heavy smokers (more than 20 pack-years) (11/30) (Additional file 2: Figure S1). *KRAS* mutations were identified in 7.4% (7/95) of *ALK* fusion-negative cases, and detected only among smokers. Though *KRAS* mutations were examined through

Table 1 Comparison of clinicopathological parameters in cases with or without ALK fusion

	with fusion	without fusion	*P*
n	35	95	
Age (years)	58.5	62.8	0.050
gender			
male	14	47	0.337
female	21	48	
smoking			
never	25	41	0.004
ever	10	54	
pStage			
I	20	60	0.532
II-IV	15	35	
differentiation grade			
wel	4	44	<0.001
mod+por	31	51	
Predominant subtype			
papillary	21	77	0.019
Acinar	13	13	
Bronchioloalveolar	0	4	
solid with mucin	0	1	
signet	1	0	
lymphatic invasion			
-	24	68	0.738
+	11	27	
Vessel invasion			
-	15	51	0.273
+	20	44	
TP53 mutation			
-	34	75	0.014
+	1	20	
EGFR mutation			
-	35	40	<0.0001
+	0	55	
KRAS mutation			
-	35	88	0.189
+	0	7	

codons 12, 13 and 61, they were found only in codon 12. The *KRAS* mutation rate increased along with the elevation of smoking index (Additional file 2: Figure S1). These findings for *EGFR* and *KRAS* mutations are consistent with previous reports from Japan, the prevalence being quite different from that in the United States [27-29].

DNA copy number alterations of chromosome arms

We compared the allelotypes of each chromosome arm between the two groups. Global views of chromosome aberrations are shown in Figure 1. Note that in *ALK* fusion-positive tumours, genomic copy number changes were more evenly distributed over the chromosome arms and high copy number gains (dark-red) in short genomic segments were less frequently encountered than with *ALK*-fusion negative examples. Significantly different patterns of respective chromosomal arm gain and loss were noted between the two groups. In fact, 5q, 8p, 9q, 11p and 11q were significantly more amplified, and 6q was more deleted in *ALK* fusion-positive tumours, whereas, in *ALK* fusion-negative tumours, 17q was more amplified, and 8p and 9p were more deleted (Figure 1, Table 2a, Additional file 1: Table S3-S5). *P*-values for comparisons of the aberration frequency in each chromosome arms are shown in Additional file 1: Table S5. When comparing global chromosome instability levels between the two groups, average numbers of chromosome arms with copy number gain or loss were 8.42 ±7.46 and 9.56 ±7.90 for tumours with and without *ALK* fusion, respectively, as detailed in Table 3, the difference not being statistically significant.

Chromosomal number alterations with advancement of pathological stage

Chromosome aberration might be expected to increase as tumours progress in stages and, if so, numbers of chromosome arms with gain and/or loss might be larger in advanced tumours. In fact however, when we compared the number of chromosome arm altered between pathological stage I and II-IV, total number did not increase in pathological stage II-IV, though only *ALK* fusion-negative tumours showing significant elevation of chromosomal gain (Figure 2).

Comparison of gain and loss frequency of selected loci

We selected twenty-one loci with recurrent copy number gain and five loci with loss to compare small-scale genomic aberrations. All the loci examined and *P*-values are summarized in Additional file 1: Table S6, S7. In Figure 3, stacked bar charts are shown indicating the

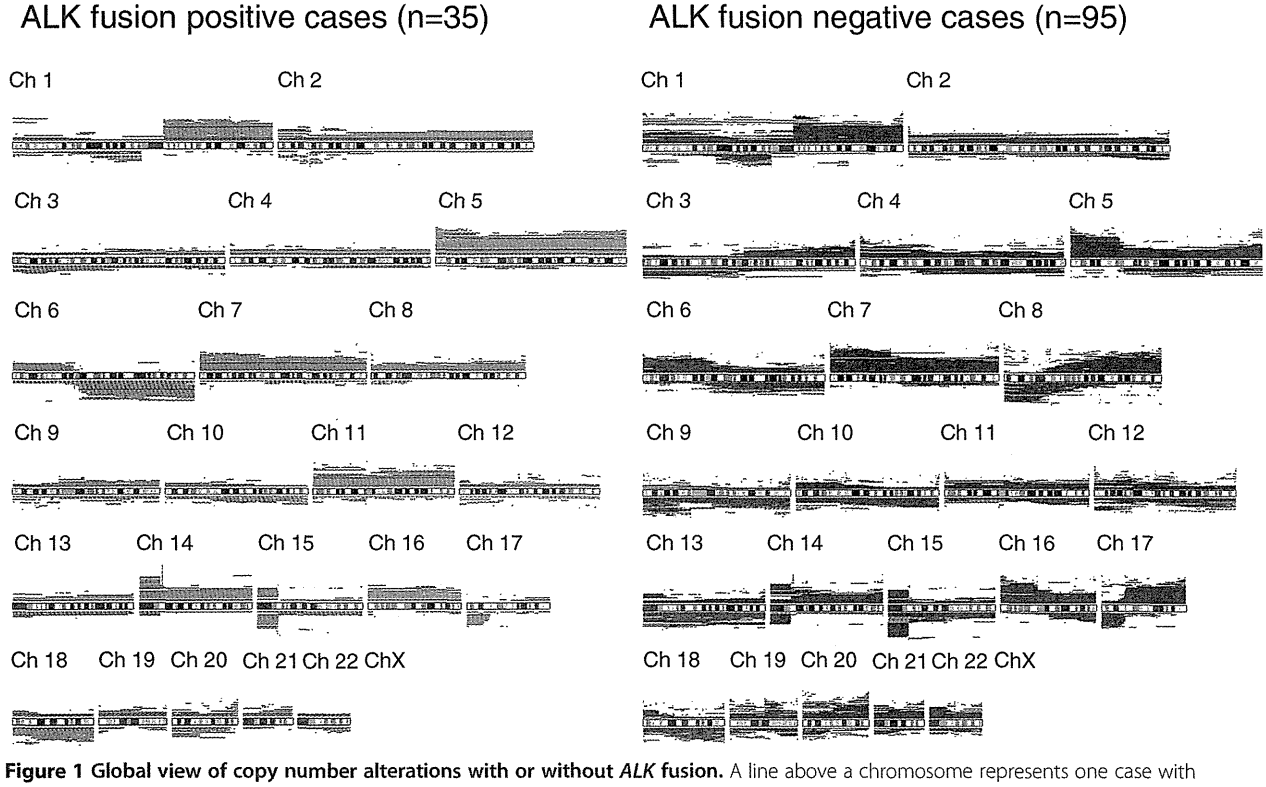

Figure 1 Global view of copy number alterations with or without *ALK* fusion. A line above a chromosome represents one case with genomic gain and its length. The color indicates the copy number of genomic regions: pink, 3 or 4 copies; and dark-red, ≥ 5 copies. Lines beneath the chromosomes represent copy number loss: blue, 1 copy; and light-green, 2 copies (homozygous deletion). Width between the lines of the two groups are adjusted according to the number of cases included for ease of visual comparison.

Table 2 Comparisons of significantly altered chromosomal arms between adenocarcinomas with and without *ALK* fusion

Category	Gain	Loss
More frequent *with* ALK fusion	5q, 8p, 9q, 11p, 11q	6q
More frequent *without* ALK fusion	17q	8p, 9q

percentage gain or loss of the selected loci. Interestingly, copy numbers (and related genes) at 1p34.3 (*MYCL1*), 7p11.2 (*EGFR*), 7p21.1, 8q24.21 (*MYC*), 16p13.3, 17q12 (*ERBB2*) and 17q25.1 were significantly less gained, and those at 9p21.3 (*CDKN2A*), 9p23-p24.1 (*PTPRD*), 13q14.2 (*RB1*) were significantly less deleted in *ALK* fusion-positive tumours than fusion-negative ones, with loci related to both oncogenes and tumour suppressor genes having fewer changes in tumours with *ALK* fusion. There were no oncogene-related loci with more gains and no suppressor gene-related loci with more losses in tumours with *ALK* fusion.

Homozygous deletions were found only at 9p21, at frequencies similar between the two groups, although the summed frequencies of heterozygous and homozygous deletions at 9p21.3 did significantly differ. In the group without *ALK* fusion, all the cases with the homozygous deletion harbored *EGFR* mutations.

MYCL1, EGFR, MYC and *ERBB2* are well-known oncogenes and *CDKN2A* and *RB1* are tumour suppressor genes related to lung carcinogenesis. *PTPRD* has been suggested to function as a tumour suppressor in several tumours, including lung cancers [30] and brain tumours [31]. Notably, 5p15.33, including *TERT* (telomerase reverse transcriptase), had the highest rate of gain in both groups regardless of *ALK* fusion (Additional file 3: Figure S2 and Additional file 1: Table S7).

Taken together, *ALK* fusion-positive tumours showed similar levels of overall chromosome instability, but when focusing on particular cancer-related regions, significantly fewer copy number gains at oncogene-related loci and significantly fewer deletions at suppressor gene-related loci.

Discussion

Recurrent chromosome translocation has been accepted to play an important role in the pathogenesis of hematological malignancies, but not of solid tumours. Recently, however, chromosome rearrangements in solid tumours such as prostate cancer and non-small cell lung cancer have been reported [32]. *ALK* fusion was originally described in anaplastic large-cell lymphoma as a chimeric protein *NPM-ALK* resulting from a translocation. More recently, evidence has accumulated that the *EML4-ALK* fusion gene defines a novel subclass of lung adenocarcinomas with distinct clinicopathological features [7-9], so that it has emerged as a target for therapy. We focused here for the first time on allelic imbalance of tumours with *ALK* fusion with a novel technique which has already shown the involvement of loss of A20 function in the pathogenesis of a subset of B-cell lymphomas [33] and gain of function of C-CBL tumour suppressor in myeloid neoplasms [34]. Applying this methodology, we demonstrated that lung adenocarcnomas with *ALK* fusion feature less amplification of loci with oncogenes and fewer deletions of loci related to tumour suppressor genes, although global chromosome aberrations were similar between tumours with and without *ALK* fusion. suggesting that the fusion gene is a driver mutation, not just a passenger mutation.

Table 3 Comparisons of numbers of chromosome arms with aberrations between adenocarcinomas with or without *ALK* fusion

	with *ALK* fusion (n=35)	without *ALK* fusion (n=95)	*P*
Gains	5.97±6.75	6.21±6.95	0.859
Losses	2.46±3.06	3.35±4.34	0.196
Total	8.42±7.46	9.56±7.90	0.454

Note that significant differences are not detected.

Genetic instability was here categorized into two groups for simplicity, at the chromosomal level and at the nucleotide level. We earlier found the former to play a more important role in lung carcinogenesis, the frequency of LOH (loss of heterozygosity) being higher in less-differentiated tumours [35]. *ALK* fusion positive tumours are more common among non-smokers and the younger population, similar to those with *EGFR* mutations. We had expected fewer chromosome aberrations in *ALK* fusion-positive tumours because tumours arising in such people usually harbor less LOH and a lower *TP53* mutation rate than smokers [36-38]. Contrary to our expectation, the global copy number changes at the chromosomal arm level did not differ between the two groups, although significant differences of alteration frequency at the individual chromosomal arms were seen. In addition, only *ALK* fusion-negative tumours showed an increase of the frequency of chromosome arm gain with the advancement of disease stage. Furthermore, at the smaller-genomic scale level, *ALK* fusion-positive tumours were less amplified at the loci containing *EGFR* family genes, 7p11.2 (*EGFR*), 17q12 (*ERBB2*) and other loci, 1p34.3 (*MYCL*), 7p21.1, 8q24.21 (*MYC*), 16p13.3 and 17q25.1. *EGFR* and *ERBB2* play important roles by dimerizing when their ligands binds to produce downward growth signals to the tumour cells. Mutations and activation of these genes may drive carcinogenesis [39], and increased expression is associated with a poor prognosis in NSCLCs [40-43]. *ALK* fusion positive tumours are speculated to be less dependent on the actions of oncogenes and tumour-suppressor genes induced by copy number changes. Our results may also indicate that there

	p-Stage	with *ALK* fusion	*P*	without *ALK* fusion	*P*
Gain	I	5.05±6.07	n.s.	4.97±6.41	0.017
	II-IV	7.20±7.59		8.53±7.43	
Loss	I	1.90±3.11	n.s.	3.60±4.66	n.s.
	II-IV	3.20±2.93		2.88±3.80	
Gain +Loss	I	6.95±6.78	n.s.	8.57±7.71	n.s.
	II-IV	10.4±8.09		11.41±8.11	

Figure 2 Comparisons of numbers of chromosome arms altered with or without *ALK* fusion in different pathological stages. Note that, whereas tumours in higher stages show more gains than stage I tumours when the tumours have no *ALK* fusion, *ALK* fusion positive tumours exhibit no such difference. p-Stage; pathological stage, n.s.; not statistically significant.

is common and frequent chromosome abnormality in lung adenocarcinomas independent of *ALK* fusion, such as the 5p15.33 region, including *TERT*.

As for genomic loss, 9p21.3 (*CDKN2A*), 9p23-p24.1 (*PTPRD*) and 13q14.2 (*RB1*) were significantly less frequently deleted in *ALK* fusion-positive tumours. Homozygous deletion was seen only at 9p21.3 including *CDKN2A* and limited to *EGFR*-mutated tumours among *ALK* fusion-negative neoplasms as reported in the literature [44] and also seen in *ALK*-fusion positive ones. That deletion of 9p23-24.1 and 13q14.2 including tumour suppressor genes was rare in *ALK* fusion-positive tumours suggests that they can grow even if the functions of these suppressor genes are retained.

Of all the selected loci, 5p15.33 containing *TERT* (telomerase reverse transcriptase isoform 2) showed the highest frequency of recurring gain regardless of *ALK* fusion. The enzyme is important for telomere regeneration and maintenance resulting in a growth advantage and Zhang et al. reported that the locus is a frequent target of amplification during tumourigenesis [45]. Copy number gain of this locus significantly correlates with telomerase activity [46] and is one of the most consistent alterations in the early stages of non-small cell lung cancer [47]. In addition, increased susceptibility to lung cancer development associated with a SNP polymorphism of this locus has been reported [48,49]. The fact that most human tumour cells have telomerase activity indicates that its acquisition is vital for carcinogenesis and cell immortalization, and it might explain the reason why lung adenocarcinomas with or without *ALK* fusion shows similar frequency of copy number gain of this locus.

Our results have some therapeutic relevance. The fact that there are less involvement of other oncogenes and tumor suppressor genes may be related to dramatic responses to targeted drugs because of intact cellular processes including apoptosis pathways. In this regard, there is an interesting paper by Camidge et al. [50], demonstrating the inverse relationship between fused and isolated red copy number on FISH might suggest

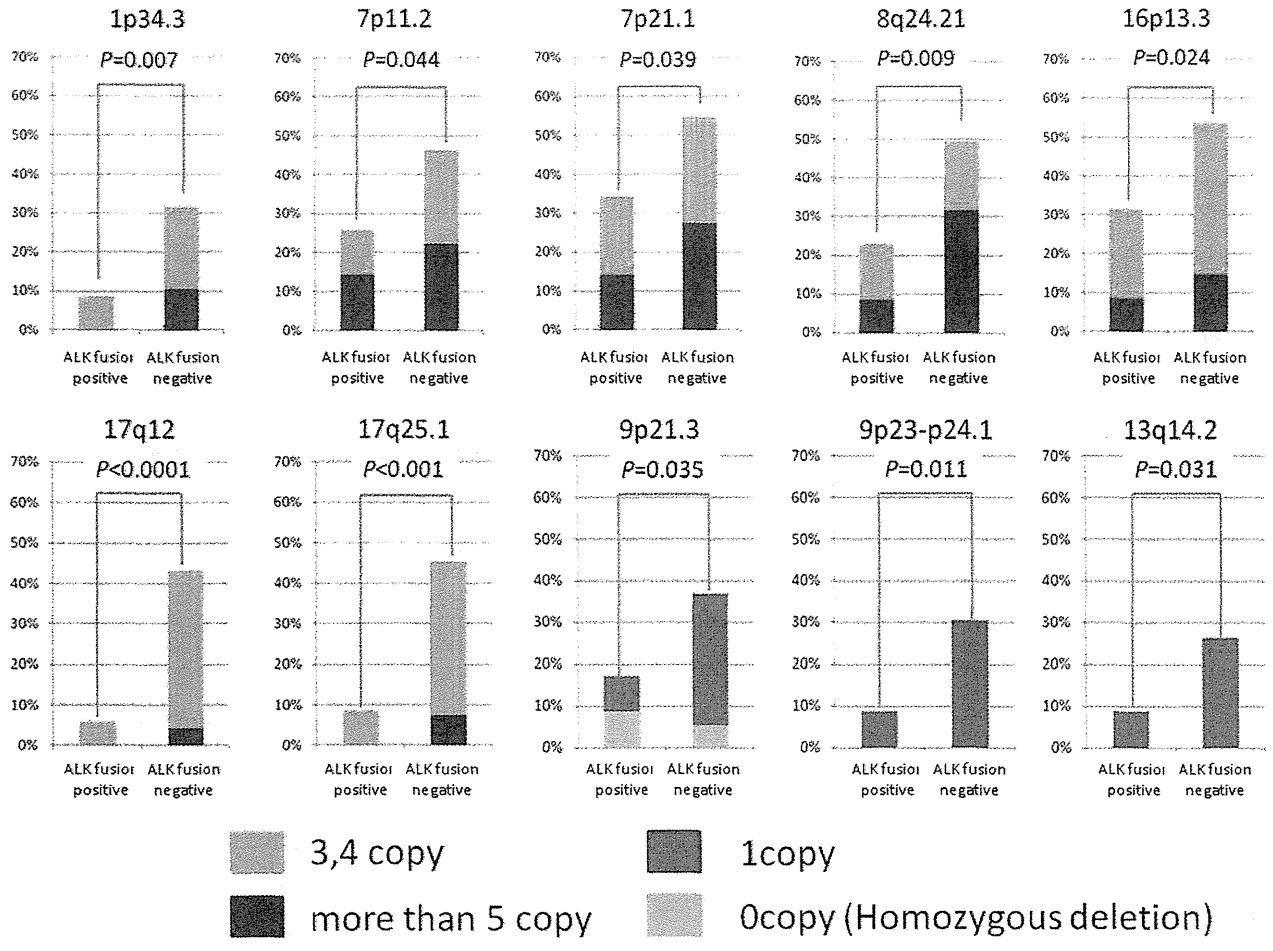

Figure 3 Significant differences in copy number change detected at seven loci for gain and three loci for loss among twenty-six selected loci. Colors of the stacked bars represent copy number: orange, 3,4 copies; red, ≥ 5 copies; blue, 1 copy loss (heterozygous deletion), light green: 2 copy losses (homozygous deletion). 17q12, 17q25.1 show remarkable differences in copy number gain between *ALK* fusion-positive and -negative tumours.

the *ALK* fusion positive tumor was a "near-diploid" subtype of non-small cell lung cancer. Comparing closely, however, between their and our results, our study clearly revealed the overall frequency of chromosome aberrations are similar between *ALK* fusion positive and negative tumors, suggesting not "near-diploid". But, certainly, we need more investigations on genomic instability of *ALK* fusion positive tumors.

It is well known that smoking causes genomic changes with allelic imbalance [20]. As shown in Table 1, smokers dominate never smokers in the group without fusion whereas the fusion-positive group has more never smokers than smokers. Since the tumors without *ALK* fusion include *EGFR*-mutated tumors, most of which are from never smokers, the *ALK* fusion-negative group is certainly heterogeneous. In due course, a study that describes comparisons of allelotypes of non-smoker's tumors between with *ALK* fusion and with *EGFR* mutation should be warranted.

Conclusions

Although overall frequencies of aberrations at the chromosome arm level do not appear to significantly differ between *ALK* fusion-positive and -negative tumors, smaller genomic regions including cancer-related genes do show significant variation. Thus tumors with *ALK* fusion feature significantly fewer copy number gains and losses at loci containing oncogenes and tumor-suppressor genes, respectively. This implies that *ALK* fusion itself exerts very strong driving forces for tumorigenesis, in other words, that *ALK* fusion is a driver mutation, not just a passenger mutation.

Additional files

Additional file 1: Table S1. Frequencies of fusion variants of *ALK* rearrangements. **Table S2.** Cases with *TP53* mutations and their smoking status. **Table S3.** Chromosomal arms and number of cases with gain with or without ALK fusion. **Table S4.** Chromosomal arms and number

of cases with loss with or without ALK fusion. **Table S5.** P-values for comparisons of the frequencies of chromosome aberrations in all chromosome arms between tumours with or without *ALK* fusion. **Table S6.** Number of cases with copy number gain or loss at selected loci with or without *ALK* fusion. **Table S7.** Significance of the differences in frequencies of copy number changes (gains and losses) between tumours with or without *ALK* fusion.

Addtional file 2: Figure S1. Mutation rates for EGFR, TP53 and KRAS according to cumulative smoking are shown. EGFR and KRAS mutations were only detected among ALK fusion negative cases, so ALK fusion positive cases were not included in the analysis. Note the gradually decrease in EGFR mutation rate with increase in cumulative smoking. KRAS mutations were detected only among smokers.

Additional file 3: Figure S2. Comparisons of copy number alteration rates at selected loci with or without *ALK* fusion. Note that 5p15.33 including *TERT* shows the highest gain both in *ALK* fusion positive and negative tumours, the frequencies being identical.

Competing interest
The authors have no potential conflicts of interest.

Authors' contributions
HN, MK, SO, HM and YI designed the study. HN, KT, KI, NM, HM and YI performed pathological and/or genomic diagnosis of tumors. HN, MK, MS and SO obtained microarray data and carried out bioinformatics analysis. HN and KN analyzed mutations. YS, SO and YI collected samples and/or provided detailed clinical data of patients. HN and YI drafted the manuscript. All authors read and approved the final manuscript.

Authors' information
HM has found ALK fusion in lung cancer with own developed cDNA library. MK, MS and SO detected genes responsible for hematological disorders through same algorithm with this study, CNAG/AsCAR. KT has created a novel diagnostic method to detect *ALK* fusion positive lung cancer. YI has found characteristic pathological features of *ALK* positive cancer.

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Diagnosis of Lung Cancer in Small Biopsies and Cytology

Implications of the 2011 International Association for the Study of Lung Cancer/American Thoracic Society/European Respiratory Society Classification

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• The new International Association for the Study of Lung Cancer/American Thoracic Society/European Respiratory Society lung adenocarcinoma classification provides, for the first time, standardized terminology for lung cancer diagnosis in small biopsies and cytology; this was not primarily addressed by previous World Health Organization classifications. Until recently there have been no therapeutic implications to further classification of NSCLC, so little attention has been given to the distinction of adenocarcinoma and squamous cell carcinoma in small tissue samples. This situation has changed dramatically in recent years with the discovery of several therapeutic options that are available only to patients with adenocarcinoma or NSCLC, not otherwise specified, rather than squamous cell carcinoma. This includes recommendation for use of special stains as an aid to diagnosis, particularly in the setting of poorly differentiated tumors that do not show clear differentiation by routine light microscopy. A limited diagnostic workup is recommended to preserve as much tissue for molecular testing as possible. Most tumors can be classified using a single adenocarcinoma marker (eg, thyroid transcription factor 1 or mucin) and a single squamous marker (eg, p40 or p63). Carcinomas lacking clear differentiation by morphology and special stains are classified as NSCLC, not otherwise specified. Not otherwise specified carcinomas that stain with adenocarcinoma markers are classified as NSCLC, favor adenocarcinoma, and tumors that stain only with squamous markers are classified as NSCLC, favor squamous cell carcinoma. The need for every institution to develop a multidisciplinary tissue management strategy to obtain these small specimens and process them, not only for diagnosis but also for molecular testing and evaluation of markers of resistance to therapy, is emphasized.

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A new lung adenocarcinoma classification has recently been published under the joint sponsorship of the International Association for the Study of Lung Cancer (IASLC), the American Thoracic Society (ATS), and the European Respiratory Society (ERS).[1] This is 1 of 2 articles that highlight major pathology-related implications of the new classification, as there are many paradigm shifts that will impact pathologists in the diagnosis and management of specimens for lung cancer.[2] As there are very different issues related to small biopsies and cytology specimens (Tables 1 and 2; Figure 1) versus resection specimens, it seemed best to address these topics in 2 separate articles.

Because 70% of lung cancers are unresectable as patients present in advanced stages, small biopsy and cytology specimens are the primary method of diagnosis for the majority of lung cancer patients. Also, prior World Health

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Table 1. Specific Terminology and Criteria for Adenocarcinoma, Squamous Cell Carcinoma, and Non–Small Cell Carcinoma, Not Otherwise Specified (NSCLC-NOS), in Small Biopsies and Cytology[a]

2004 WHO Classification, Including Updated IASLC/ATS/ERS Terminology	Morphology/Stains	IASLC/ATS/ERS Terminology
Adenocarcinoma	Morphologic adenocarcinoma patterns clearly present	Adenocarcinoma (describe identifiable patterns present)
Mixed subtype		
Acinar		
Papillary		
Solid		
Micropapillary		
Lepidic (nonmucinous)		Adenocarcinoma with lepidic pattern (if pure, add note: an invasive component cannot be excluded)
Lepidic (mucinous)		Invasive mucinous adenocarcinoma (describe patterns present; use term mucinous adenocarcinoma with lepidic pattern if pure lepidic pattern; see text)
No 2004 WHO counterpart; most will be solid adenocarcinomas	Morphologic adenocarcinoma patterns not present (supported by special stains, ie, +TTF-1)	Non–small cell carcinoma, favor adenocarcinoma
Squamous cell carcinoma	Morphologic squamous cell patterns clearly present	Squamous cell carcinoma
No 2004 WHO counterpart	Morphologic squamous cell patterns not present (supported by stains, ie, +p40)	NSCLC, favor squamous cell carcinoma
Large cell carcinoma	No clear adenocarcinoma, squamous or neuroendocrine morphology or staining pattern	NSCLC-NOS[b]

Abbreviations: IASLC/ATS/ERS, International Association for the Study of Lung Cancer/American Thoracic Society/European Respiratory Society; NSCLC, non–small cell lung carcinoma TTF-1, thyroid transcription factor-1; WHO, World Health Organization.

[a] Modified with permission from Travis et al.[1] The new IASLC/ATS/ERS international multidisciplinary lung adenocarcinoma classification. *J Thorac Oncol.* 2011;6(2):244–285.

[b] NSCLC-NOS pattern can be seen not only in large cell carcinoma but also when the solid, poorly differentiated component of adenocarcinoma or squamous cell carcinoma is sampled but does not express immunohistochemical markers or mucin.

Organization (WHO) classifications primarily addressed resection specimens,[3,4] so they did not propose standardized terminology and criteria for small biopsies and cytology. Therefore, this article addresses one of the most important aspects of this classification. Although the IASLC/ATS/ERS classification primarily addressed lung adenocarcinoma, because no formal terminology or criteria were proposed for small biopsies and cytology, this classification provides for the first time a proposed set of terms and criteria for all major histologic types of lung cancer in these types of specimens.

MAJOR CHANGES IN PATHOLOGY ARE DRIVEN BY ADVANCES IN THORACIC ONCOLOGY

Largely driven by therapeutic advances, a revolution is taking place in the lung cancer field that has major implications for pathologic diagnosis and tissue management. The new IASLC/ATS/ERS classification was devel-

Table 2. IASLC/ATS/ERS Classification for Small Biopsies/Cytology Comparing 2004 WHO Terms With New Terms for Small Cell Carcinoma, Large Cell Neuroendocrine Carcinoma (LCNEC), Adenosquamous Carcinoma, and Sarcomatoid Carcinoma[a]

2004 WHO Classification	Small Biopsy/Cytology: IASLC/ATS/ERS
Small cell carcinoma	Small cell carcinoma
LCNEC	Non–small cell carcinoma with NE morphology and positive NE markers, possible LCNEC
Large cell carcinoma with NE morphology	Non–small cell carcinoma with NE morphology (negative NE markers) Comment: This is a non–small cell carcinoma where LCNEC is suspected, but stains failed to demonstrate NE differentiation.
Adenosquamous carcinoma	Morphologic squamous cell and adenocarcinoma patterns present: non–small cell carcinoma, NOS (comment that adenocarcinoma and squamous components are present and this could represent adenosquamous carcinoma).
No counterpart in 2004 WHO classification	Morphologic squamous cell or adenocarcinoma patterns not present but immunostains favor separate glandular and adenocarcinoma components Non–small cell carcinoma, NOS (specify the results of the immunohistochemical stains and the interpretation) Comment: this could represent adenosquamous carcinoma.
Sarcomatoid carcinoma	NSCLC with spindle and/or giant cell carcinoma (mention if adenocarcinoma or squamous carcinoma are present)

Abbreviations: IASLC/ATS/ERS, International Association for the Study of Lung Cancer/American Thoracic Society/European Respiratory Society; NE, neuroendocrine; NOS, not otherwise specified; NSCLC, non–small cell lung carcinoma; WHO, World Health Organization.

[a] Reprinted with permission from Travis et al.[1] The New IASLC/ATS/ERS international multidisciplinary lung adenocarcinoma classification. *J Thorac Oncol.* 2011;6(2):244–285.

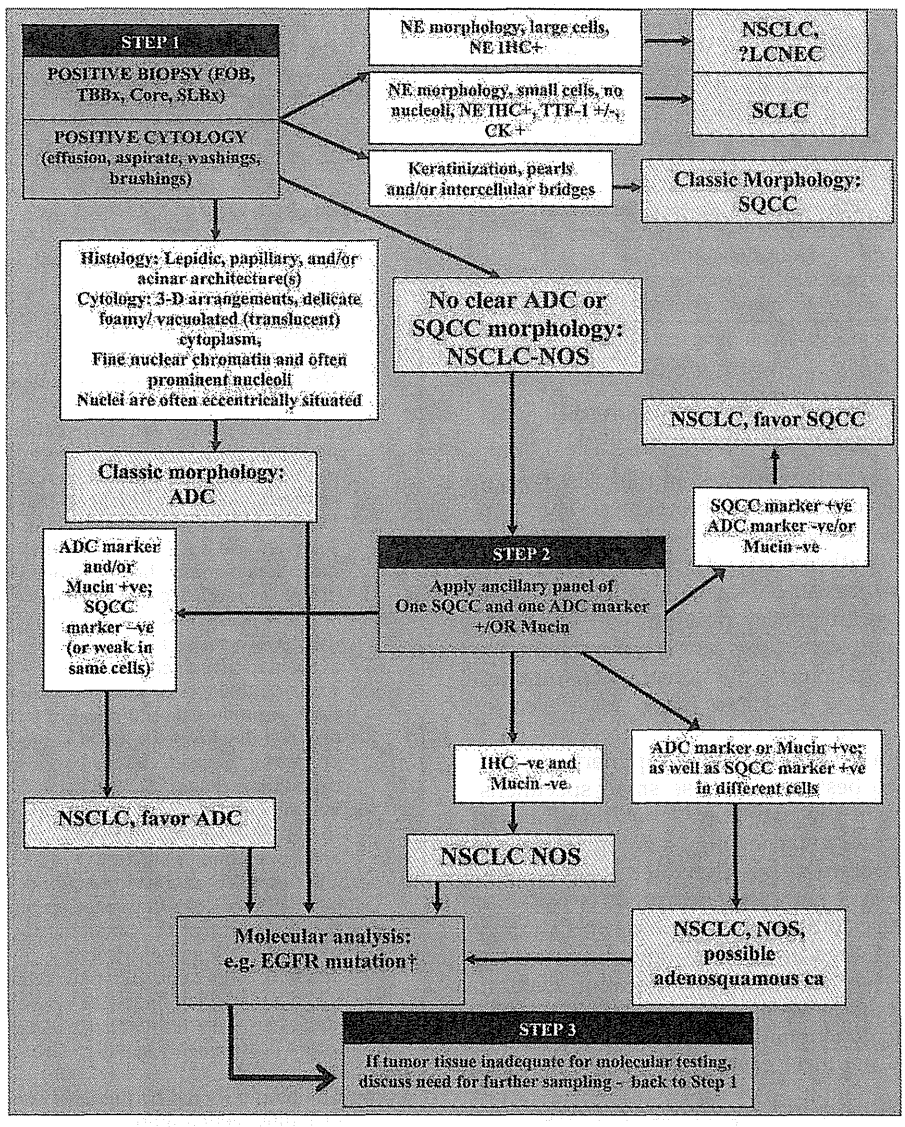

Figure 1. *Step 1: When positive biopsies (fiberoptic bronchoscopy [FOB] or transbronchial [TBBx], core, or surgical lung biopsy [SLBx]) or cytology (effusion, aspirate, washings, brushings) show clear adenocarcinoma (ADC) or squamous cell carcinoma (SQCC) morphology, the diagnosis can be firmly established. If there is neuroendocrine morphology, the tumor may be classified as small cell carcinoma (SCLC) or non–small cell lung carcinoma (NSCLC), probably large cell neuroendocrine carcinoma (LCNEC) according to standard criteria. If there is no clear ADC or SQCC morphology, the tumor is regarded as NSCLC, not otherwise specified (NOS). Step 2: NSCLC NOS can be further classified based on (1) immunohistochemical stains, (2) mucin (diastase–periodic acid-Schiff or mucicarmine) stains, or (3) molecular data. If the stains all favor ADC, with positive ADC marker(s) (ie, thyroid transcription factor 1 [TTF-1] and/or mucin positive) and negative SQCC markers, then the tumor is classified as NSCLC, favor ADC. If SQCC markers (ie, p63 and/or cytokeratin [CK] 5/6) are positive with negative ADC markers, the tumor is classified as NSCLC, favor SQCC. If the ADC and SQCC markers are both strongly positive in different populations of tumor cells, the tumor is classified as NSCLC-NOS, with a comment it may represent adenosquamous carcinoma. If all markers are negative, the tumor is classified as NSCLC-NOS. See text for recommendations on NSCLCs with marked pleomorphic and overlapping ADC/SQCC morphology. † Epidermal growth factor receptor (EGFR) mutation testing should be performed in (1) classic ADC; (2) NSCLC, favor ADC; (3) NSCLC-NOS; and (4) NSCLC-NOS, possible adenosquamous carcinoma. In these cases, if EGFR mutation testing is negative, testing for EML4-anaplastic lymphoma kinase (ALK) should be performed. In NSCLC-NOS, if either EGFR mutation or ALK rearrangements are positive, the tumor is more likely to be ADC than SQCC. Step 3: If clinical management requires a more specific diagnosis than NSCLC-NOS, additional biopsies may be indicated.*

Abbreviations: ca, carcinoma; IHC, immunohistochemistry; NE, neuroendocrine; +, positive; –, negative; +/–, positive or negative; –ve, negative; +ve, positive.

oped by an international multidisciplinary panel including pathologists, medical oncologists, respiratory physicians, radiologists, molecular biologists, and thoracic surgeons to address some of these issues.[1] It also was based on a systematic review to weigh evidence and make recommendations (Table 3).[1,5] In this document, the evidence-based recommendations are listed with the strength of the recommendation and quality of the evidence according to the grades of recommendation, assessment, development, and evaluation method (Table 3).[6] In addition, some recommendations are provided for good clinical practice (Table 4). Some research recommendations are also made in areas of uncertainty (Table 5). For this article, we have selected the recommendations taken from the main classification publication that are pertinent to the diagnosis of lung cancer in small biopsy and cytology specimens.

Table 3. Summary of Pathology Recommendations Applicable to Small Biopsy and Cytology Specimens

1. For small biopsies and cytology, we recommend that NSCLC be further classified into a more specific type, such as adenocarcinoma or squamous cell carcinoma, whenever possible (strong recommendation, moderate quality evidence).
2. We recommend that the term NSCLC-NOS be used as little as possible, and we recommend it be applied only when a more specific diagnosis is not possible by morphology and/or special stains (strong recommendation, moderate quality evidence).

Abbreviations: NSCLC, non–small cell lung carcinoma; NOS, not otherwise specified.

Multidisciplinary Approach Is Required for Lung Cancer Diagnosis

Many of the new concepts presented in this classification are the direct result of the multidisciplinary approach, which includes clinicians, molecular biologists, radiologists, and surgeons and pathologists. One of the central proposals in this classification is that lung cancer diagnosis is now clearly a multidisciplinary problem. All specialists involved with the diagnosis of lung cancer patients need to work closely together to achieve the correct diagnosis and to obtain appropriate and sufficient tissue for molecular testing.

Each institution must have a multidisciplinary strategy that addresses how to best obtain these small specimens, how to process them in the pathology laboratory, how to preserve material for molecular testing, sending specimens to the molecular laboratory for expedited testing, and reporting the results in a pathology report. It is useful to have a multidisciplinary committee to develop this strategy and to keep lines of communication open in order to monitor issues as they arise in an ongoing fashion. Pathologists should take a leadership role in this process. Because there are widely varying institution-specific issues, this should be set up at a local level.

Personalized Medicine in Lung Cancer Is Driven by Histologic Cell Type and Genetics

Now that lung cancer therapy is becoming personalized for individual patients based on the histologic cell type and subtypes of lung cancer (adenocarcinoma versus squamous) and molecular status (ie, epidermal growth factor receptor [*EGFR*] mutation and anaplastic lymphoma kinase [ALK] rearrangement in adenocarcinoma), the pathologist's role and approach to lung cancer diagnosis in small biopsies and cytology has been affected dramatically. Specific therapies are selected for patients depending on the histologic diagnosis and the molecular status of the tumor. Understanding this new concept is essential for pathologists as they manage these specimens.

In particular, there have been 4 therapeutic advances for non–small cell lung carcinoma (NSCLC) since the 2004 WHO classification. These changes are directly tied to precise histologic classification. The first relates to tyrosine kinase inhibitors as first-line therapy in patients with advanced lung adenocarcinoma with *EGFR* mutations.[7–11] Second, adenocarcinomas with ALK rearrangements are responsive to crizotinib.[12–14] Third, patients with adenocarcinoma or NSCLC, not otherwise specified (NSCLC-NOS), are more responsive to pemetrexed than those squamous cell carcinoma.[15–17] Fourth, squamous cell carcinoma is associated with life-threatening hemorrhage in patients treated with bevacizumab; therefore, it is contraindicated in lung cancer patients with this histology.[18]

Based largely on multiple phase III clinical trials,[7–11] the following clinical recommendation was made in the new classification.

Table 4. Summary of Pathology Considerations for Good Practice Applicable to Small Biopsy and Cytology Specimens

1. When a diagnosis is made in a small biopsy or cytology specimen in conjunction with special studies, it should be clarified whether the diagnosis was established based on light microscopy alone or if special stains were required.
2. The term *non–SQCC* should not be used by pathologists in diagnostic reports. It is a categorization used by clinicians to define groups of patients whose tumors comprise several histologic types and who can be treated in a similar manner; in small biopsies/cytology, pathologists should classify NSCLC as ADC, SQCC, NSCLC-NOS, or other terms outlined in Table 1 or Figure 1.
3. The above strategy for classification of ADC versus other histologies and the terminology in Table 1 and Figure 1 should be used in routine diagnosis as well as future research and clinical trials, so that there is uniform classification of disease cohorts in relation to tumor subtypes and data can be stratified according to diagnoses made by light microscopy alone versus diagnoses requiring special stains.
4. Tissue specimens should be managed not only for diagnosis but also to maximize the amount of tissue available for molecular studies.
5. To guide therapy for patients with advanced lung ADC, each institution should develop a multidisciplinary team that coordinates the optimal approach to obtaining and processing biopsy/cytology specimens to provide expeditious diagnostic and molecular results.
6. When paired cytology and biopsy specimens exist, they should be reviewed together to achieve the most specific and concordant diagnoses.
7. The terms AIS and MIA should not be used for diagnosis of small biopsies or cytology specimens. If a noninvasive pattern is present in a small biopsy, it should be referred to as a lepidic growth pattern.
8. The term large cell carcinoma should not be used for diagnosis in small biopsy or cytology specimens and should be restricted to resection specimens where the tumor is thoroughly sampled to exclude a differentiated component.
9. Cell blocks should be prepared from cytology samples including pleural fluids.
10. In biopsies of tumors that show sarcomatoid features (marked nuclear pleomorphism, malignant giant cells, or spindle cell morphology), these should be initially classified as according to guidelines above in relation to ADC; NSCLC, favor ADC; SQCC; or NSCLC favor SQCC, as this is apt to influence management, with additional statement that giant and/or spindle cell features (depending on what feature) are present. If such features are not present, the term NSCLC-NOS should be used, again with comment on the sarcomatoid features.
11. Neuroendocrine immunohistochemical markers should be performed only in cases where there is suspected neuroendocrine morphology. If neuroendocrine morphology is not suspected, neuroendocrine markers should not be performed.

Abbreviations: ADC, adenocarcinoma; AIS, adenocarcinoma in situ; MIA, minimally invasive adenocarcinoma; NOS, not otherwise specified; NSCLC, non–small cell lung carcinoma; SQCC, squamous cell carcinoma.

Table 5. Pathology Research Recommendations Applicable to Small Biopsy and Cytology Specimens

1. It is unknown whether there is any added value provided by refining NSCLC-NOS via immunohistochemistry on small biopsies or cytology samples. This requires assessment in future trials using systemic therapy.
2. Additional markers for squamous or adenocarcinoma differentiation, such as desmoglein-3[102] or desmocollin[103] for squamous cell carcinoma or napsin A for adenocarcinoma[103], need further evaluation.

Abbreviation: NSCLC-NOS, non-small cell lung carcinoma, not otherwise specified.

Clinical Recommendation

In patients with advanced lung adenocarcinoma, we recommend testing for *EGFR* mutation (strong recommendation, moderate quality evidence).

Remarks: This is a strong recommendation because potential benefits clearly outweigh harms. This recommendation assumes that correct classification by *EGFR* mutation status is associated with important benefit based on randomized phase III clinical trials of EGFR tyrosine kinase inhibitor therapy that demonstrate a predictive benefit for response rate and progression-free survival, but not overall survival,[7–11] as well as subset analyses of multiple additional studies.

This clinical recommendation is listed in this document because of the major impact this has on the role for pathologists, not only in diagnosis but also in management of tissue for molecular testing. Now, not only do pathologists need to make a correct diagnosis, but also they need to manage the small amounts of cells and tissue in a manner that will preserve as much as possible for molecular testing.

Identification of New Molecular Targets in Lung Cancer Is a Rapidly Evolving Field

There are several examples of rapid advances occurring in the discovery of molecular targets for novel therapies in lung cancer.

An excellent example is the discovery that crizotinib is a clinically effective ALK inhibitor in patients with locally advanced or metastatic non–small cell lung cancer.[12,14] This was recently approved by the Food and Drug Administration for use in this setting: if the tumor is ALK positive as detected by a Food and Drug Administration–approved test or the Vysis ALK Break-Apart fluorescence in situ hybridization probe kit (Abbott Molecular, Des Plaines, Illinois).[12,14] Other methods of detection such as immunohistochemistry show promise to be reliable methods of detecting ALK rearrangements,[19–21] but these need to be tested and validated in clinical trials. Although the Food and Drug Administration approval for crizotinib occurred after publication of the IASLC/ATS/ERS lung adenocarcinoma classification,[22] testing for ALK rearrangement is now part of molecular diagnostic testing for lung adenocarcinomas. The efficacy of crizotinib is now in need of further validation in phase III clinical trials. Anaplastic lymphoma kinase gene rearrangements are mostly found in lung adenocarcinomas lacking *EGFR* or Kirsten rat sarcoma (*KRAS*) mutations, and they are frequently thyroid transcription factor 1 (TTF-1) positive.[23,24]

ROS1 rearrangement was recently described in 1.7% of lung adenocarcinomas, and it appears to identify another subset of lung adenocarcinoma patients for whom there be an effective molecular targeted therapy.[25,26] ROS1 rearrangements are mutually exclusive with ALK rearrangements and also tend to occur in young never smokers with the histology of adenocarcinoma. There does not appear to be an association with a specific histologic subtype. One patient had a near complete response to crizotinib.[26]

A frequent complication of EGFR tyrosine kinase inhibitor therapy is the development of acquired resistance through acquisition of *EGFR* T790M mutations, cMET amplification, dedifferentiation of the tumor with epithelial-mesenchymal transition, or development of a small cell carcinoma component.[27–30] For this reason, additional biopsies may be indicated in patients who have tumor progression after an initial response to tyrosine kinase inhibitor therapy. This phenomenon is also being observed with ALK inhibitors and is likely to occur with other molecular targeted therapies as well.[12]

There is also promise for lung squamous cell carcinoma with the recent discovery that fibroblast growth factor receptor 1 (FGFR1) amplification and discoidin domain receptor tyrosine kinase 2 (*DDR2*) mutations may render these patients sensitive to FGFR1 inhibition and dasatinib respectively.[31–33] Also, the Cancer Genome Atlas (TCGA) project sponsored by The National Cancer Institute has identified molecular alterations that may represent molecular targets in over 60% of squamous cell carcinomas of the lung.[34]

As a result of these advances, therapeutic decisions are now based on tumor typing by histology and/or cytology. This is leading to major changes in how pathologists diagnose lung cancer in small biopsy and cytology specimens. Therefore, pathologists need to make a greater effort to separate adenocarcinoma from squamous cell carcinoma; this includes a limited workup with special stains such as immunohistochemistry or mucin stains.[1,35] Although currently there is a rationale for molecular testing for *EGFR* mutation and ALK rearrangement in tumors classified as adenocarcinoma; NSCLC, favor adenocarcinoma; or NSCLC-NOS, it is anticipated that specific molecular tests will soon be recommended in squamous cell carcinomas, perhaps for FGFR-1 amplification or *DDR2* mutation.

These recent advances indicate that pathologists involved with lung cancer diagnosis need to pay close attention to the literature to be aware when molecular advances have reached the point of sufficient validation to be introduced into clinical practice. This is a challenge for practicing pathologists, because there are many new markers that are being recognized, but they may be neither ready nor suitable for routine clinical practice.

MAJOR CHANGES IN NEW CLASSIFICATION

Major changes in the approach to classification of lung cancer are introduced in the new IASLC/ATS/ERS classification compared with previous WHO classifications: (1) greater use of special stains to classify difficult cases further into adenocarcinoma or squamous cell carcinoma, (2) diagnosis using small samples, and (3) the need to manage tissue strategically for molecular studies. Several changes in terminology and introduction of new entities are addressed more fully in the second article, which focuses on classification of adenocarcinoma in resection specimens. These relate to the discontinuation of the terms bronchioloalveolar carcinoma and adenocarcinoma, mixed subtype, as well as the introduction of micropapillary as a new histologic subtype, the term lepidic pattern for the former bronchioloalveolar carcinoma growth pattern, and the specific term invasive mucinous adenocarcinoma for overtly