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Defining morphologic features of invasion in pulmonary non-mucinous adenocarcinoma with lepidic growth
- A proposal by theIASLC Pathology Committee

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Abstract

Background: Since the 8th edition of UICC/AJCC TNM classification system the primary tumor pT stage is determined based on presence and size of the invasive components. The aim of this study was to identify histological features in tumors with lepidic growth pattern that may be used to establish criteria for distinguishing invasive from non-invasive areas.

Materials and Methods: A Delphi approach was used with two rounds of blinded anonymized analysis of resected non-mucinous lung adenocarcinoma cases with presumed invasive and non-
invasive components, followed by one round of reviewer de-anonymized and unblinded review of cases with known outcomes. A digital pathology platform was used for measuring total tumor size and invasive tumor size.

**Results:** The mean coefficient of variation for measuring total tumor size and tumor invasive size was 6.9% (range 1.7-22.3%) and 54% (range 14.7-155%), respectively, with substantial variations in interpretation of the size and location of invasion among pathologists. Following the presentation of the results and further discussion among members at large of the IASLC Pathology Committee, extensive epithelial proliferation (EEP) in areas of collapsed lepidic growth pattern is recognized as a feature likely to be associated with invasive growth. EEP is characterized by multilayered luminal epithelial cell growth, usually with high grade cytological features in several alveolar spaces.

**Conclusion:** Collapsed alveoli and transition zones with EEP were identified by the Delphi process as morphologic features that were a source of interobserver variability. Definition criteria for collapse and EEP are proposed to improve reproducibility of invasion measurement.
Introduction

Primary lung adenocarcinomas show diverse histologic appearances and substantial intratumoral heterogeneity in growth pattern. According to the 4th (2015) and 5th (2021) Editions of WHO classification of non-mucinous lung adenocarcinoma, the predominant pattern is used for subtyping the tumors and is the basis for the proposed grading system for surgically resected adenocarcinomas. Furthermore, it is suggested that the proportions of each pattern be recorded at 5% increments. The concepts of adenocarcinoma in situ (AIS) and minimally invasive adenocarcinoma (MIA), first established in the 2015 WHO classification, are now recognized more frequently, particularly in patients diagnosed in lung cancer screening protocols. Both AIS and MIA are associated with 100% 5-year survival after complete resection and essentially no metastatic risk. In contrast, tumors with at least 5 mm (effective diameter) of invasive pattern disease (≥pT1a) have been associated with recurrence risk that increases with the extent of invasion.

The importance of recognizing and distinguishing lepidic growth from other patterns regarded as “invasive” became highly relevant for pT staging in the 8th edition of UICC/AJCC TNM classification system, which recommended that in non-mucinous lung adenocarcinoma the primary tumor size (pT) is determined by the invasive size excluding the lepidic component, which is considered non-invasive. The IASLC Staging and Prognostic Factor Committee also encouraged further research on what is the best method and reproducibility of measuring size of invasive versus lepidic components and how this could be improved.

Currently, pathologists are recommended to measure the maximum diameter of the invasive patterns, or estimate the percentage of invasive patterns relative to overall size to calculate invasive size for pT staging. However, reproducibility in distinguishing different patterns of growth and recognition of invasion remain challenging in more than occasional cases of resected adenocarcinoma, especially when there is iatrogenic collapse of the alveolar framework, and particularly in distinguishing lepidic from papillary, acinar or even micropapillary patterns.

As a follow-up to the research questions posed in the IASLC lung cancer staging proposal on assessment of tumor size in part-solid tumors, the IASLC Pathology Committee formed an
Invasion Working Group to revisit the issue of recognizing areas of invasion in non-mucinous lung adenocarcinoma. The aim was to examine the reproducibility of invasive size measurement and identify histological features that may be used to establish criteria in distinguish invasive from non-invasive patterns in resected lung non-mucinous adenocarcinomas showing a lepidic component, especially in those cases prone to iatrogenic collapse. To this end the Delphi approach was used, which is a relevant source of evidence in health care research.\textsuperscript{13–15}

**Materials and methods**

An overview of the studies performed is shown in Figure 1. Two different study sets, comprising resected lung adenocarcinoma cases, were used in this work. The first contained tumors regarded by the contributing pathologist to represent histological invasive and non-invasive adenocarcinomas (total cases n=32, pathologists n=22). The second set (total cases n=28, pathologists n=27) included 9 cases selected for the presence of lymph node metastases or recurrence as a proof of invasion, and 19 cases thought by the contributing pathologist to have no evidence of invasion at diagnosis and found to have no clinical evidence of recurrence/metastases at follow-up. The reviewers were blinded to nodal status and outcome data. Institutional ethics approvals for the use of materials in this study were obtained by contributing pathologists at their respective hospitals.

Standard histological slides were prepared from one representative formalin-fixed paraffin-embedded (FFPE) block and stained with hematoxylin-eosin (H&E), elastin stain and cytokeratin 7 immunohistochemical stain. The slides were scanned and made available from a server at the University of Tsukuba, Japan, similarly to what previously described,\textsuperscript{16} but for this study developed by Frontier System Co. Ltd (Mito, Ibaraki, Japan). For each case in the first step of evaluation only the whole slide H&E image was available for reading. In the second step, the H&E and elastin stains were available together for evaluation. In the third step, cytokeratin 7 stain was then added. After each step, the pathologist had to decide, based on the 2015 WHO classification,\textsuperscript{3} whether any invasive carcinoma was present and choose one of the options: i) invasive, ii) non-invasive or iii) ‘do not know’. Subsequently, the pathologists were asked to provide total tumor size and “invasive” tumor size measurement by
using a digital ruler tool. For the second cohort, also blinded to outcome, the pathologists were also asked to draw a line to indicate the location of invasion. One measurement line was obligatory, but the viewer could choose to include up to two additional lines to locate the invasive areas. The line(s) were recorded for subsequent analysis. This set allowed identification of possible invasive and (non)invasive morphological characteristics.

Following the completion of slide review of both cohorts 1 and 2, tumor and invasive size measurements were revealed and the clinical outcomes were unblinded to a subset of pathologists in a study working group. This working group used this information to identify features that might potentially be useful to distinguish non-invasive lepidic pattern from other invasive patterns. In this phase, the lines drawn were de-anonymized as to observer, and the outcome unblinded. Importantly, the group focused on pT1 cases with a defined endpoint of nodal metastasis to deconstruct the criteria for invasion seen in those cases using a Delphi procedure. Expert diagnosis from a previous round as well as the reasons for their judgments were evaluated in a meeting of the invasion working group. It is believed that during this process the range of the answers will decrease and the group will converge towards the "correct" answer. The results were presented for discussion to members at large of the IASLC Pathology Committee on February 29th, 2020. For this meeting the participants received their own data, but not other pathologists’ data. Further iterations of refinement took place virtually until July 2021. A flow chart was developed to encapsulate the deconstructed diagnostic process with elements in common amongst the group or which emerged from discussion, see Figure 2. The results of working group deliberation and a drafted manuscript was distributed to the Pathology Committee members at large for review and further discussion on September 22, 2021.

Criteria testing
To assess the utility of the flowchart elements, a third, image-based, validation set of 43 images was sent to the 10 invasion working group members. Image magnification and size was comparable to the previous interobserver study. In this validation phase, participants recorded their initial morphologic impression regarding invasion and scored as present or absent those criteria identified following the Delphi meeting as supporting or refuting invasion: altered alveolar architecture (tumor induced alteration versus iatrogenic collapse), extensive epithelial
proliferation (EEP), desmoplasia, interstitial growth, high nuclear grade, nuclear shape from cuboidal to columnar or pleomorphic, visible transition in cytologic appearances, (absence of) luminal alveolar macrophages between putative non-invasive and invasive areas (Table 1). EEP is characterized by multilayered epithelial cells, usually showing high grade cytological features (enlarged nuclei, increased nuclear-to-cytoplasmic ratio, nuclear pleomorphism) lining alveolar spaces, which would otherwise be considered as lepidic pattern disease (see Figure 3). The panelists recorded their initial subjective or gestalt impression and coded each feature as present (1) or absent (0), where score of 1 favored invasion. Major criteria for invasion were the presence of EEP, desmoplasia and altered alveolar architecture. The sum of the major criteria (up to a score of 3) was compared to the initial subjective impression. The maximum number of pathologists with the same score (categorized in 0-1 vs 2-3) was used to calculate the concordance percentage.

**Statistical analysis**

For each case the mean value, standard deviation and coefficient of variation (across pathologists) of total tumor size and invasive size was calculated by the study statistician (BLW).\(^\text{17}\) Cases were ranked for the number of invasive diagnoses. For comparison of coefficient of variation the modified signed-likelihood ratio test\(^\text{18}\) in the R cvequality package was used. This test for comparison coefficient of variations at case level was applied on total size and invasive size measurements as well as for comparison of coefficient of variations between one invasion size measurement and the sum of 2 or 3 lines. The criteria testing was evaluated with dichotomous McNemar test (≧ 7 pathologists same score; invasion: gut feeling “yes” /≧ 2 out of 3 criteria “present”). A p-value <0.05 was considered to be significant.

**Results**

**Tumor size measurements**
For measuring total tumor size in the first cohort, the mean coefficient of variation among pathologists was 6.9% (range 1.7-22.3%, see Supplementary Figure 1A). In contrast, when measuring tumor invasive size, the mean coefficient of variation was 54% (range 14.7-155%; Supplementary Figure 1B). A casewise comparison of coefficient of variation between both measurements revealed a significant difference in almost all cases (p<0.001), except for 1 case (p=0.34). The majority of the pathologists used 1 line for the designation of the invasive area. On average two pathologists (range 0-11) used 2 lines and on average two pathologists (range 0-6) used 3 lines to designate invasive areas. The coefficient of variation was not significantly different for comparison between one or up to 3 lines for measurements of invasion.

Graphical displays of each case summarizing the areas of invasion, drawn by the 27 observers for cohort 2, showed a frequent notable difference in interpretation of the size and location of invasion (see Figure 4). The distribution of pathologists’ scores for invasive versus non-invasive is shown in Supplementary Figure S2. A pT category was assigned for this distribution if a category had 3 or more pathologists (>10%) out of 27 pathologists scores. The distribution of pathologic tumor (pT) categories in the second cohort is shown in Supplementary Table S1. There was a marked difference in pT categorization in 26 out of the 28 cases under assessment. Following this analysis, an effort to identify morphologic features driving individual decisions to recognize areas as either “invasive” or “non-invasive” resulted in the following considerations, which are based on the cohort of cases with established invasive behavior (i.e., lymph node metastases) and those with long follow-up.

**Morphological consideration in defining features of invasion**

Morphological features that might lead to a more consistent designation of ‘lepidic (non-invasive)’ versus ‘not lepidic’ are proposed in Table 1.

**The effect of iatrogenic collapse**

During the Delphi discussion, compression of the alveolar structure/lepidic pattern also called surgical collapse\(^{19}\) or iatrogenic collapse\(^{20}\) was recognized as a frequent phenomenon in pulmonary resection specimens (Supplementary Figure S3). This pattern may affect the shape of normal alveoli as well as alveoli lined by tumor cells and can significantly modify the microscopic appearance of the tumor. It was acknowledged that collapse and compression of
alveolar structures, especially when thickened by increased stroma with chronic inflammatory cell infiltrate and lined by tumor cells, may lead to collapsed lepidic pattern showing folding and tufting that mimic papillary, micropapillary or acinar architectures when cross-sectioned. It was recognized that perfusion fixation through the airways and/or transpleural perfusion by needle and syringe may reduce the amount of artefactual collapse, and thus may assist in identification of the collapsed lepidic pattern. However, in many cases, this process may not fully mitigate this collapse artifact.

**Features favoring invasion**

Cases were categorized as having “definite evidence for invasion” when conventional morphological criteria for invasion could be identified, including effacement of alveolar architecture (Figure 5A) and stromal invasion characterized by desmoplastic stroma infiltrated by single or small nests of tumor cells and/or vascular (Figure 5 B, C), bronchial/ bronchiolar wall (Figure 5 D, E) or pleural invasion. Desmoplastic stroma defined as collagenous response in relation to invasion with morphologically loose fibromyxoid stroma containing fibroblasts (neofibrogenesis/fibroplasia) was frequently, but not always, seen in combination with invasive tumor cells in invasive adenocarcinomas (Figure 5). Fibroelastosis alone was not considered sufficient for invasion. The presence of an occasional subepithelial area of fibromyxoid stroma was interpreted with caution, as this can be seen in organizing pneumonia and idiopathic pulmonary fibrosis. Nonetheless, when prevalent within the lesion, desmoplastic stroma is a useful marker of invasive disease.

Strikingly, in cases with the above-described features of ‘definite evidence for invasion’, as well as in some cases lacking conventional invasion criteria, a feature the group descriptively termed ‘extensive epithelial proliferation’ (EEP) was consistently noted (Figure 5 B-E). EEP is characterized by multilayered epithelial cells, usually showing high grade cytological features (enlarged nuclei, increased nuclear-to-cytoplasmic ratio, nuclear pleomorphism) lining alveolar spaces, which would otherwise be considered as lepidic pattern disease. EEP is a category of growth that exceeds what could be considered as non-invasive disease. Areas that lack conventional invasion criteria frequently include proliferation that falls short of criteria for micropapillary pattern.
Sometimes EEP may involve the whole lesion. As such, EEP is considered a cellular feature associated with invasion when architectural features of invasion are indeterminate. It is therefore not a ‘new’ adenocarcinoma pattern used for grading but instead one to answer a binary question of lepidic or non-lepidic when definitive architectural features are lacking. However, as indeterminate architecture is often found adjacent to established invasive patterns, assignment of such areas to the invasive versus non-invasive lepidic pattern was a major source of interobserver differences. It was acknowledged that EEP was a subjective assessment, but since it could not be readily explained by tissue compression or cutting artefacts only, it could serve as an independent criterion of invasive transition. To improve consistency, stratification of two or more cells was proposed to define EEP.

**Features favoring lepidic growth with collapse**

Non-invasive characteristics were observed in one or more of the cases and could be contrasted with definite evidence of invasion elsewhere in the lesion. These features included: (1) presence of collapsed/compressed peripheral (alveolar) lung tissue lined predominantly by a single layer of monotonous cells; (2) luminal (alveolar) spaces arranged in a regular manner, often with the long axes of the spaces arranged in parallel (parallel streaming) (Supplementary Figure 4 and 5, see also Figure 5 F-K) and (3) an abrupt transition of monolayered tumor cells to type I pneumocytes at the periphery of the lesion, with a continuation of the orientation of compressed or collapsed non-neoplastic alveoli. Recognition of this final pattern helped distinguish lepidic non-invasive from acinar invasive disease, especially when collapse was associated with an increase in fibroelastotic interstitium and shrinkage of alveolar space diameter26. Alveolar macrophages were not infrequently found in collapsed spaces and when abundant, could influence the shape of the adjacent collapsed alveolar walls (Figure 5 H-K). However, in some cases with EEP alongside loose luminal epithelial cells, CK7-negative morphologic alveolar macrophages could also be discerned. Thus, the presence of alveolar macrophages alone, in what appears to be an airspace, is not an absolute criterion for non-invasive pattern of disease, assuming acceptance of EEP as a surrogate marker for invasion.

‘Uninformative’ features
Histologic features that were considered not informative to distinguish between invasive and non-invasive areas (possible pitfalls) are listed in Supplementary Table 2 and shown in Supplementary Figure S4.

**Angulated glands**

In contrast to many other organs where angulated spaces or glands lined by tumor cells (glands) may be frequently observed in invasive adenocarcinomas, in pulmonary adenocarcinomas angulated spaces are frequently present in both invasive and in collapsed, non-invasive lepidic proliferations (Figure 5K). An area with fibroelastotic or mature fibrotic scar, as distinct from neofibroplasia/desmoplastic reaction, is not considered as evidence to support invasion (Supplementary Figure S6). Therefore, angulated or round gland-like structures without evidence of desmoplasia are not informative for the distinction between invasive and non-invasive areas.

Similarly, in some cases, the pre-existing lepidic growth close to the scar may become either angulated and/or much reduced in size whilst maintaining a rounded shape. Ultimately, these may be reduced to a tiny focus comprising less than 10 cells, a pitfall which may be compounded by tangential sectioning. The context of such a finding (lack of neofibroplasia and presence of larger and more obvious lepidic areas) helps avoid an erroneous diagnosis of invasion (acinar pattern).

**Alveolar septal thickening**

Alveolar septal thickening is frequently seen in association with neoplastic epithelial proliferations without other evidence of invasion and is not of itself considered a morphologic criterion for invasion (Supplementary Figure S7). The thickening may be due to i) infiltration with inflammatory cells; ii) fibrosis or iii) increase in elastin (e.g. as in Noguchi type B27). In lepidic proliferations with \( \leq 2 \) cell layers (without other invasive patterns) and the presence of cancer associated fibroblasts, 100% 5 years survival rate has been reported28.

**Cytomorphology**
Variation in cytomorphology (tumor cell atypia) can be difficult to interpret. At one extreme, it is usual, in the lepidic pattern or adenocarcinoma in situ, for the cytology of the tumor cell population to be relatively low grade and sometimes of hobnail morphology. However, this is not absolute, as invasive disease may be cytologically low grade, whilst lepidic pattern disease can demonstrate a range of cytological grade, some low grade, some high grade. In tumors where both lepidic and non-lepidic components are present, a transition of cytological features may be helpful in making a distinction between the two components of the lesion (Figure 5M). This may also help in borderline cases of EEP. For example, in a predominantly lepidic pattern lesion, with low grade hobnail cytology, ‘acini’ lined by the same population are likely to be collapsed lepidic foci, whilst spaces lined by larger, more pleomorphic cells with larger nuclei and more eosinophilic cytoplasm probably represent invasive disease, even in the absence of associated neofibrogenesis. On the other hand, the group also recognized that in some cases, lepidic pattern disease may show mixed cytomorphology, some areas of low grade (presumed pre-existing in situ) disease, whilst other areas are high-grade, similar to the invasive components. As such, cytologic change alone without EEP was not considered sufficient criteria for invasion.

Inconclusive issues
Although the above-described morphological criteria were helpful in the distinction between invasive and non-invasive areas, some cases had areas with inconclusive findings and are summarized in Table 3. Furthermore, in the whole or part of the resection specimen, delayed or inadequate fixation may lead to sloughing off epithelial cells from the basement membrane. Care has to be taken not to interpret this as sign of micropapillary pattern of invasion.

Diagnostic algorithm.
In daily practice a pathologist will frequently form a first impression about a possible diagnosis which is an initial subjective impression (also called gut feeling or ‘gestalt’ impression). However, an analysis based on reproducible criteria may supersede this impression and reduce interobserver variability. A flow chart has been constructed, shown in Figure 2, to aid in the consistent identification of invasion in pulmonary adenocarcinoma. To determine the utility of the flowchart elements described above, a set of 43 images (see Supplementary Figure S8) was
scored by the 10 members of invasion working group to assess inter-observer concordance. After excluding one member’s uninterpretable responses, the remaining 9 members had an average concordance of initial subjective impression of 79%. The criteria-based scores for the major criteria (EEP, desmoplasia and altered alveolar architecture) improved concordance to 84%. Accepting a score of ≥ 7 out of 9 as a concordant case, the number of concordant cases increased from 25 (58%) for initial subjective impression to 34 (79%) using the three major criteria (Supplementary Table S 2 and Supplementary Figure S9; McNemar test 0.049). The inclusion of minor criteria did not change the agreement rate.
Discussion

For pathologic staging of non-mucinous adenocarcinoma, the measurement of invasive size is required. The large variation in these measurements, especially in adenocarcinomas that at least partly grow along alveolar walls, prompted a search for more detailed histological criteria to guide the identification of invasion. A table and flowchart (see Table 1 and Figure 2) evolved with a practical suggestion for day-to-day practice, with the aim to make the decision of invasion versus no invasion more consistent. It is, however, recognized that these remain proposals focused on improved reproducibility that require wider validation.

The moderate reproducibility of invasive pattern recognition in adenocarcinomas has been shown by the pathology committee publication of the IASLC in 2012. In that study one high magnification static image per case was used for classification, where some pathologists were more inclined towards invasion than others, with a kappa score of 0.55±0.06 and 0.08±0.02, for easy and difficult cases, respectively. The current study was performed on digitized slides, reflecting a closer approximation of daily practice of small adenocarcinomas by allowing a broader assessment of the tumor, and yet again shows that there is major room for improvement. The appearance of differences around interpretation of invasion between four studies on small adenocarcinomas is also shown in Supplementary table S3. Moreover, another study used, instead of the histological patterns for assessment of invasion, an alternative approach defined as a lung adenocarcinoma without nodal involvement, vascular invasion, or lymphatic invasion, implying that the WHO classification was not followed in this respect. These examples of variation in interpretation and usage of the actual managerial classification highlight the need for improvement in the assessment of invasion in pulmonary adenocarcinoma.

In the literature a previous interobserver study on measurement of invasion revealed (quote): “good agreement between (two) observers when classifying tumors as AIS, MIA, and invasive adenocarcinoma” and “significant differences in overall survival between the 3 groups for both observers, and interobserver variability was evident”. This contrasts with our study and is explained by several factors. I) The composition of the cases in that study comprised 296 nodules
of which 59% were, in hindsight, agreed invasive adenocarcinomas (including 11% stage III and IV), while our study has a focus on pT1 adenocarcinomas. II) Recalculating their data for the remaining 41% of the cases reveals 52/123 (42%) discordant cases, of which the largest part is a difference between MIA and invasive adenocarcinoma, similar to our study. III) Their study was performed by 2 pathologists from one institute, while ours involved up to 27 pathologists from different parts of the world, probably reflecting a more global performance, and disclosing a marked variability. It is clear from our study that, in applying the WHO classification, there are marked differences in assigned areas of invasion (54% coefficient of variation), as graphically displayed in Figure 4. However, if the object is clear (limits of tumor edge), the measurement can be quite precise (7% coefficient of variation for total tumor size).

This raises two major questions: Why does so much interobserver variability exist and how can it be improved? The canonical classifications in oncopathology are hybrids of managerial classification grids superimposed on histogenetic classification. Tumor classification and diagnosis is a community activity and the expert plays an essential regulatory role in that community. In assigning different categories to tumor classification, the reproducibility of the categories or phenomena used for distinction in categories should be high enough to expect generalizability (external validity) throughout the world. The issue of invasion in pulmonary adenocarcinoma appears to be associated with too low kappa score to rely upon in a managerial classification. The minimum acceptable value of a kappa score, or alternative ways to express reproducibility, is, however, not known.

Finding solutions to such a problematic issue is difficult. As the issue evolved while applying the approach formulated in the WHO classification of lung cancer, a possible solution required an ‘out of the box thinking’ approach. We used a Delphi procedure to the extent that we did not reach the phase of a recommendation for implementation, but developed a concept for further study by the IASLC pathology community. A first step in the evaluation of our cases revealed that classical morphological criteria of invasion, such as pleural, bronchial and vascular invasion and desmoplastic stroma with individual cells or small clusters are not always recognizable in cases with lymphogenic and or haematogenic metastases. However, in cases with established invasive behavior, the presence of EEP, defined as two or more tumor cell layers growing on the
luminal side of the alveolar basement membranes could frequently be discerned even in a background of potentially retained alveolar architecture. In strict morphological/biological terms, EEP may not reflect an invasive focus in and of itself, but rather may represent a surrogate for invasive potential and is frequently observed in association with other characteristics of true invasion. The Delphi process at a minimum revealed it as an ambiguous pattern leading to discordance in the assessment of invasive size. In an effort to improve invasive size reproducibility, the expert panel agreed to arbitrarily include EEP within the invasive size measurement, however this designation requires further independent validation.

This is a recognition that EEP should only be used for separation of invasive versus non-invasive areas and not for assigning into one of the currently recognized patterns. When these areas were adjacent to what was agreed upon as lepidic, and often in a zone between lepidic and classic invasive patterns, nuclear grade in the EEP was often higher than the adjacent definitive lepidic component, more closely resembling that in the unambiguous invasive portion. According to the concept of EEP (see also Figure 3, the lower end of the category does not meet criteria for papillary, micropapillary, acinar or solid growth, while the higher end approaches these conventional patterns of invasion, raising the possibility that EEP represents partial involvement of an alveolar space by a tumor clone with invasive properties. EEP is defined as a luminal epithelial proliferation with stratification of two or more cells. Further investigation is needed about this threshold. Also, the extent of stratification needs to be analyzed to avoid inclusion of folds and tangential cutting. During the writing of this document, Yotsukura and coworkers published a study with emphasis on cancer associated fibroblasts and also demonstrated that a lepidic pattern with 2 or less cell layers was associated with a 100% 5 years survival. Whether this is the optimal threshold as opposed to 2, 3 (or more) cell layers requires further study.

The review of images by the panel by initial subjective impression and then scoring of criteria was informative. Major criteria, when seen in combination, modestly improve the agreement of observers in invasive versus non-invasive designation, thus supporting their use in assessing invasive size for staging. The use of the minor criteria was not directly supported; however, these remain in the flowchart as their utility may be greater in whole slides than in static images,
as they pertain to areas of transition. We recognize that this tool for determination of invasion in pulmonary adenocarcinoma may not be explicit in every single case. The minor criteria may be of value when the major criteria are more ambiguous.

In case of doubt whether an area should be designated as invasive or non-invasive, the majority of the group decided in favor of “upgrading”: i.e., after taking into account all of the morphological factors described above, in cases where there remained doubt as to whether or not invasion was present, we favor concluding that the doubtful area be considered invasive disease. Part of the motivation for this decision is that the overall measurement of the tumor grossly and histologically is reproducible, and in the absence of definitive reason should not be superseded. We are aware that this contradicts the recommendations in the TNM Staging system, where cases are down-staged, when there is doubt. The conclusion of the majority of the invasion working group is an attempt to be pragmatic and reflect prevailing diagnostic practice.

The switch from *in situ* to invasive tumor growth represents a crucial stage in the evolution of lung adenocarcinoma. However, the biological understanding of this shift is limited. In some cases, it represents evolution of an invasive subclone, but sometimes, as demonstrated by Moore and colleagues\(^{29}\) the *in situ* component represents peripheral outgrowth of invasion-competent disease rather than a pre-existing low-grade precursor. The classic appearance of invasiveness arising in a low-grade lesion was characterized by: (i) clear nuclear grade difference between the *in situ* and invasive components, (ii) architectural asymmetry reflecting the stochastic appearance of the invasive component; and (iii) the absence of an *in situ* ‘penumbra’, with penumbra referring to an *in situ* component of uniform width at the edge of the lesion. The cytonuclear change (transition) is also presented in Table 1.

In the cases where it is difficult to judge the presence of invasion the pathologist should err on the side of invasion. This may also diminish the fear of possible underdiagnosis and understaging.

Several issues may modify the morphology of pulmonary adenocarcinoma. Firstly, the recognition of iatrogenic collapse is important. Although the 5th edition of the WHO classification mentions “parenchymal collapse” on page 68\(^1\), a difference between the pathological (irreversible) collapse and iatrogenic (at least partly reversible) collapse is not made\(^{20}\). A sense of the magnitude of the diagnostic difference is obtained from a small proof of
principle study, examining the effect of iatrogenic collapse on adenocarcinoma classification: in
around 20% of the cases the diagnosis was downgraded to AIS\textsuperscript{40}. Although perfusion fixation
through the airways and/or transpleural perfusion by needle and syringe may reduce the amount
of artefactual collapse in many cases, this mitigation effort may not be fully realized: i) wedge
and larger resections may be sectioned fresh for frozen section analysis; ii) despite perfusion
fixation, collapse can still be an issue: the lobe volume reached, after perfusion fixation, on
average 50% of the lobe volume calculated with CT imaging\textsuperscript{41}; iii) in some countries, like
Japan\textsuperscript{42}, and some laboratories in the UK, Netherlands\textsuperscript{43}, Switzerland and USA, perfusion
fixation is part of the routine handling, whilst in many other countries this is not the case. The
group agreed that perfusion fixation is recommended for lung resections, when possible,
recognizing that this may be difficult in situations requiring fresh tumor tissue procurement for
clinical trials and correlative research.

A second issue may be the effect of pre-analytical handling. The routine formaldehyde fixation is
usually adequate in small biopsies, but especially in larger surgical specimens it may be delayed
due to low diffusion rate of formalin, especially if fixation depends on immersion in, rather than
inflation with, the fixative\textsuperscript{20}. Forcing samples into too small containers with insufficient formalin
amounts also induces parenchymal compression and inadequate fixation artifacts. This is a
frequent occurrence for those laboratories receiving samples from external hospitals and already
fixed in formalin. In addition, while reduction of ischemic time has been a focus for breast
specimens, leading to more rapid delivery to pathology, this is not routine in lung specimens.
The detachment of the respiratory epithelial cells from the basement membrane is frequently
seen in autopsy specimens. Likewise, tumor cells undergo the same delay in fixation and may
become detached\textsuperscript{43}. Recognition of poorly fixed areas is important, as these areas with the
misleading appearance should not be considered during diagnostic assessment, if possible.

The use of an ancillary stains such as the elastin\textsuperscript{20} and cytokeratin 7 stain for recognition of the
pre-existing alveolar frame work / underlying lobular architecture was discussed, see
Supplementary Table S4 and Supplementary Figure S10. The majority of the group felt that
more studies are needed.
A major limitation of this study is the lack of validation in a large independent cohort. The addition of a new feature to a classification may also add further confusion\textsuperscript{37}. In this paper we have tried to understand the factors influencing the subjective judgements made by a large group of experienced pulmonary pathologists when assessing early-stage adenocarcinomas. Although the IASLC Staging and Prognostic Factor Committee encouraged further research on what is the best method of measuring size of invasive versus lepidic components\textsuperscript{7}, we used a ruler available in the software to measure invasive size and register invasive area, but did not attempt to determine what the best method is for establishing invasive area. A one-dimensional ruler is a validated approach for measuring the maximum axis/diameter of an area\textsuperscript{44}. Nevertheless, all measurements in this study were conducted with the same method, providing validity for the poor reproducibility on measurements of invasion by pulmonary pathologists from all over the world.

We have identified a number of features, including consistently increased cellularity in a setting of retained alveolar architecture, which we have called EEP, and which appears to lead to a more consistent and accurate discrimination between the binary question of invasive versus non-invasive growth of lung adenocarcinoma. This work and the proposals within require validation and study in other large cohorts; we hope the wider lung pathology community will take up this challenge.
Acknowledgement

Dr. I. Wistuba’s leadership as chair of the IASLC pathology committee by creating an invasion working group is greatly appreciated. The support of Casey Connolly from the IASLC with manuscript communication is greatly appreciated.
References


38. Marchevsky AM, Walts AE, Lissenberg-Witte Bl, Thunnissen E. Pathologists should probably forget about kappa. Percent agreement, diagnostic specificity and related


Legends

Figure 1
Graphical overview of studies performed

Figure 2.
A flow chart for the thought processes for establishment of invasion in pulmonary adenocarcinoma.

Figure 3.
Examples of iatrogenic collapsed lung are shown without (A, B) and with the category of EEP (B-F) from slight (C, D) to micropapillary (E, F), cribriform (G, arrows) and solid (H, arrow) growth.

Figure 4.
Two examples of adenocarcinoma cases where lines denoting the invasive space as assigned by different observers. In case A, 21 of the 27 observers judged this case as invasive and 6 as non-invasive. In case B 9 of the 27 observers judged this as non-invasive. Note the remarkable difference in line size and location of assigned invasive areas and also realize that several observers did not interpret these cases as invasive: i.e. did not add a line.
Figure 5.
Morphological appearances observed in an adenocarcinoma

An overview of adenocarcinoma in resection specimen with partly iatrogenic collapse and also fixed by bronchial perfusion as example of reduced invasive size compared to total tumor size. (A) The tumor with blue circle is demonstrating invasive area. (B) Higher magnification of left rectangle in (A) with luminal extensive epithelial proliferation (EEP) category and (C) invasive acinar pattern around a pulmonary artery. (D) Higher magnification of right rectangle in (A) with luminal EEP and invasive growth in bronchial mucosa (E). (F) Part of iatrogenic collapsed lung with monolayer of tumor cells, which are less atypical than in EEP. (G) luminal smoker’s macrophages. (H) iatrogenic collapsed lung with visceral pleura on the left and and (I) higher magnification. (J) Transition of collapsed lung to non-malignant alveolar walls. Visceral pleura on the left side. (K) and (L) shows detail of (J). The yellow boxes in (K) show focal areas with tangential cutting leading to seemingly multilayering, which are not interpreted as EEP. The yellow lines in (K) intend to accentuate the more or less parallel orientation of the collapsed lumina (streaming pattern). Note i) the streaming pattern in (H) and (J) and ii) also that the luminal collapse is less prominent when filled with e.g. alveolar macrophages. (M) Area with transition between low (upper and lower left of yellow lines) and (on the right of the yellow lines) high grade cytology. All images H&E stain.

(N) Other adenocarcinoma case with desmoplastic stroma around acinar carcinoma
In (O) a line of 8 mm denoting the invasive part (compatible with the oval in A) in the section while total tumor size measures 18 mm.

Thought process: areas like in F-L do i) not have conventional signs of invasion; ii) not have EEP, desmoplastic stroma or interstitial growth; iii) not have high grade cytology, but fit in collapsed AIS with partial streaming, monolayer of low grade tumor cells and alveolar macrophages. Angulation may be part of the collapse of alveolar tissue and when covered with epithelial tumor cells may still be considered lepidic disease (see e.g. 5K). Likewise, in collapsed lepidic disease, structures may appear round and lack a prominent streaming pattern. These areas are not considered to be invasive should not be part of the measurement for invasive size.
Table 1. Histological features supporting the distinction between invasive and non-invasive areas.\(^{A)}\)

<table>
<thead>
<tr>
<th>Criterion</th>
<th>Description</th>
<th>Invasion</th>
<th>Not Invasion</th>
</tr>
</thead>
<tbody>
<tr>
<td>WHO</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Invasive pattern</td>
<td>Acinar, Papillary, Micropapillary, Solid</td>
<td>Present</td>
<td>Absent or uncertain</td>
</tr>
<tr>
<td>Definitive lepidic pattern</td>
<td>Monolayer alveolar growth without collapse</td>
<td>Absent or uncertain</td>
<td>Present</td>
</tr>
<tr>
<td>IASLC Major</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Extensive epithelial proliferation (EEP)(^B))</td>
<td>A possible lepidic pattern, but with luminal epithelial multilayered proliferation</td>
<td>Present</td>
<td>Absent</td>
</tr>
<tr>
<td>Altered pre-existing alveolar architecture</td>
<td>Due to invasive tumor growth(^C)</td>
<td>Present</td>
<td>Absent (^D))</td>
</tr>
<tr>
<td>CollapsE(^E)</td>
<td>Iatrogenic collapse with AIS parallel (streaming) pattern</td>
<td>Absent</td>
<td>Present</td>
</tr>
<tr>
<td>Desmoplastic stroma(^F)</td>
<td>Fibromyxoid stroma around tumor cells</td>
<td>Present (^G))</td>
<td>Absent</td>
</tr>
<tr>
<td>Interstitial growth(^H)</td>
<td>Growth of malignant cells within the stroma of alveolar walls</td>
<td>Present</td>
<td>Absent</td>
</tr>
<tr>
<td>IASLC Minor(^I)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cytology(^J)</td>
<td>Nuclear grade(^K)</td>
<td>High grade</td>
<td>Low grade</td>
</tr>
<tr>
<td></td>
<td>Nuclear shape</td>
<td>Pleomorphic</td>
<td>Monotonous</td>
</tr>
<tr>
<td>Cytologic transition(^K)</td>
<td>Cells in putative invasive area have higher nuclear grade than those of adjacent lepidic pattern</td>
<td>Higher grade than adjacent lepidic pattern</td>
<td>NA</td>
</tr>
<tr>
<td>Luminal alveolar macrophages</td>
<td>Macrophages in lumen of collapsed spaces</td>
<td>Absent(^L))</td>
<td>Present</td>
</tr>
</tbody>
</table>

\(^A)\) Beside classic histologic criteria of invasion (pleural, vascular, bronchial invasion, lymph node metastases).
B) Defined as a luminal epithelial proliferation beyond a monolayer that is 2, 3 or more layers in thickness. Optimal threshold needs to be defined in further studies. This may also be cribriform or solid [luminal filling].

C) In the space beside alveoli: individual tumor cells, small acinar glands (smaller than alveolar size)

D) Altered pre-existing architecture by (pre-existing) disease other than malignancy e.g. emphysema may also show in-situ growth.

E) Iatrogenic collapse may show parallel orientation, but is not obligatory, as local circumstances determine the actual folding of the alveolar walls such as luminal filling by alveolar macrophages, fibrosis of the alveolar wall, bronchovascular bundle in the vicinity, etc. The presence of collapse does not exclude the presence of true invasion elsewhere, see e.g. Figure 5. In adenocarcinomas with tumor cell growth along alveolar walls, this may also undergo iatrogenic collapse.

F) Desmoplastic stroma is defined as fibromyxoid stroma with admixed tumor cells. Fibroblastic foci without tumor cells may be present in AIS.

G) Not present in all patterns of invasion (e.g. micropapillary)

H) Growth of malignant cells within the stroma of easily recognizable alveolar walls with lack of malignant cells on the alveolar wall lining, is an uncommon sign of invasion. This pattern of invasion may also be seen in metastases from other organs 45.

I) If major criteria are not present, the cytology may be helpful, but not decisive. More studies needed.

J) In the context of pre-existing architecture EEP is usually accompanied with high grade cytology. Without multilayering in the whole lesion, high grade cytology alone in the context of pre-existing architecture is not sufficient for invasion. A change of low to high grade cytology is frequently associated with other signs of invasion.

K) Clear nuclear grade difference (size/shape/pleomorphism) between in situ and invasive components have been mentioned by Moore and colleagues in invasive tumours 29.

L) Alveolar macrophages are usually absent in EEP but may be present, e.g. in areas with micropapillary growth.
Table 2. Histologic criteria that are not informative for the distinction between invasive and non-invasive areas.

<table>
<thead>
<tr>
<th>Criterion</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Angular ‘glands’ without desmoplasia A)</td>
<td>Tumor gland-like structures with sharp angles may also be present in non-invasive areas.</td>
</tr>
<tr>
<td>Round ‘glands’ without desmoplasia</td>
<td>In collapsed alveolar structure with lepidic disease, spaces lined by epithelium may appear round without evidence of the streaming pattern. Usually these are also small, with a proportionate increase in fibroelastosis.</td>
</tr>
<tr>
<td>Inflammation</td>
<td>Stromal lymphocytic, plasmacellular infiltrate</td>
</tr>
<tr>
<td></td>
<td>Neutrophilic granulocytes between tumor cells</td>
</tr>
<tr>
<td>Alveolar wall thickness</td>
<td>Pre-existing alveoli covered with tumor cells</td>
</tr>
</tbody>
</table>

A) Tumor glands with sharp angles may be observed in invasive adenocarcinomas of many organs. In collapsed lepidic (in situ) growth, angulated glands are frequently present.
Table 3. Features that may confound the interpretation of invasion.

<table>
<thead>
<tr>
<th>Pre-analytic</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Poor fixation</td>
<td>Delay in fixation may also lead to insufficient/poor fixation recognized by detached cells mimicking micropapillary clusters(^{46}) as is frequently seen in autopsy specimen.</td>
</tr>
<tr>
<td>Deflation</td>
<td>Prominent deflation leads to iatrogenic collapse of alveolar tissue (Figure 2) which may lead to inappropriate classification of invasive patterns</td>
</tr>
<tr>
<td>MICROSCOPIC examination</td>
<td>The unavoidable need to obtain a histological section from a non-uniform 3-dimensional framework, requires cross cutting, and may lead to:</td>
</tr>
<tr>
<td>Equivocal architecture</td>
<td>Formation of acinar-like or papillary-like structures that mimic invasion, especially when the interstitium is fibrotic. Parallel streaming may be present to aid in distinction between true invasion and tangential cutting, but areas of uncertainty may remain. Loss of a regular pattern of tumor lined spaces (not regular/parallel enough for reliable designation of lepidic disease), but also insufficient for invasion.</td>
</tr>
<tr>
<td>Luminal cells</td>
<td>The appearances of both single cells lying freely within alveoli and multilayering (&gt;1 cell thick) of the epithelium. Interpretation is necessarily subjective though features related to tangential cutting tend to be focal within acini rather than circumferential.</td>
</tr>
<tr>
<td>Stratification</td>
<td>Nuclear / cellular stratification of epithelial tumor cells involving only part of the alveolar space circumference, where the remainder of the ‘space’ is lined by single layers of cells. This may be a biological phenomenon, but also due to tangential cutting.</td>
</tr>
</tbody>
</table>
Cohort 1
- Cases (scanned slides) n=32
- Pathologist n=22

Recording
- Total tumor size
- Invasive size [mm], by observer
- Mark invasive area by 1 line

Cohort 2
- Cases (scanned slides) n=28
  - Proven invasion* n=9
  - Indefinite for invasion** n=19
  - Pathologists n=27

Recording
- Total tumor size
- Invasive size [mm], by observer
- Mark invasive area by up to 3 lines

* Lymph node metastasis n=8; recurrence n=1
** Follow-up time/case shown in supplemental figure S2

<table>
<thead>
<tr>
<th>Step</th>
<th>Slide</th>
<th>Output</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>HE whole image</td>
<td>Diagnosis (one choice):</td>
</tr>
<tr>
<td>2</td>
<td>HE + elastic</td>
<td>1. Invasive</td>
</tr>
<tr>
<td>3</td>
<td>CK7</td>
<td>2. Non-invasive</td>
</tr>
<tr>
<td>4</td>
<td>HE whole slide image</td>
<td>3. Do not know</td>
</tr>
<tr>
<td>5</td>
<td>Invasive size and location</td>
<td>Largest tumor diameter</td>
</tr>
<tr>
<td></td>
<td>(cohort 1: draw 1 line)</td>
<td>(cohort 1: draw 1 line)</td>
</tr>
<tr>
<td></td>
<td>(cohort 2: draw 1-3 lines)</td>
<td>(cohort 2: draw 1-3 lines)</td>
</tr>
</tbody>
</table>

Results and analysis

Delphi procedure
- Invasion working group n=10

Topics of discussion
- Review study results (cohort 2)
- Iatrogenic collapse
- Histological criteria of invasion
- Possible new features of invasion

Feature validation
- Screen shots (×10x) n=43
- Working group members n=10

Recording
- "Gut feeling": + or – for invasion
- Individual features: present/absent
Flow chart for determination of invasion in pulmonary adenocarcinoma

**Primary Criteria**

- Is there invasion of pleura, blood vessel or airway?
  - No
  - Possible
  - Is there solid, micropapillary, papillary or acinar pattern\(^1\) ?
    - No
    - Possible
    - Can the focus represent pre-existing alveolar architecture\(^2\)\(^3\) ?
      - Yes
      - Possible
      - Is there extensive epithelial proliferation?
        - No
        - Possible
        - Is there desmoplastic stroma or alveolar interstitial tumor growth?
          - Yes
          - In conjunction with above answers as “possible”
          - may support invasion
          - No Invasion
          - Diagnosis
          - Invasion

\(^1\) Elastin stain may be useful
\(^2\) Cytokeratin stain may be useful

**Supportive Criteria**

- Is there high grade cytology with cytologic transition?
  - Yes or possible
  - In conjunction with above answers as “possible”
  - may support invasion
  - No
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B. Lissenberg-Witte, Alain Borczuk, Erik Thunnissen: Data curation;
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Alain Borczuk, E. Thunnissen: Visualization;
Mary Beth Beasley, Alain Borczuk, Sanja Dacic, Keith M Kerr, Yuko Minami, Andrew G Nicholson, Masayuki Noguchi, Lynette Sholl, Ming-Sound Tsao, E. Thunnissen: Roles/Writing - original draft;