αvβ6 integrin may be a potential prognostic biomarker in Interstitial Lung Disease

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MeSH terms: integrin alpha(v)beta(6), Idiopathic pulmonary fibrosis, VATS, Immunohistochemistry

Word Count

Abstract: 198 words

Manuscript: 3290 words

What is the key question?

Are immunomarkers useful for predicting prognosis in fibrotic interstitial lung diseases?

What is the bottom line?

High levels of $\alpha v\beta 6$ integrin immunostain on VATS biopsy predict worse survival than low levels of immunostain.

Why read on?

This paper describes the first immunomarker to be associated with survival in fibrotic interstitial lung diseases and describes a strategy that could alter management of these diseases.

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ABSTRACT

Background

Idiopathic Pulmonary Fibrosis (IPF) and fibrotic Non-Specific Interstitial Pneumonitis (NSIP) are progressive Interstitial Lung Diseases (ILDs) with limited treatment options and poor survival. However, the rate of disease progression is variable, implying there may be different endotypes of disease. We hypothesised immunophenotyping biopsies from ILD patients might reveal distinct endotypes of progressive fibrotic disease, that may facilitate stratification when undertaking clinical trials for novel therapies for IPF.

Methods

43 paraffin-embedded, formalin fixed lung tissue sections were immunostained for five molecules implicated in the pathogenesis of the fibrosis: alpha smooth muscle actin (αSMA); αvβ6 integrin; pro-surfactant protein c (pro-SpC); hepatocyte growth factor (HGF) and tenascin-c (TenC). Levels of immunostaining and numbers of fibroblastic foci were quantified using operator dependent and independent methods. The relationship of all these markers to overall survival was analysed.

Results

Staining revealed high levels of αSMA, αvβ6 integrin, pro-SpC, HGF and Ten C, and fibroblastic foci. Immunostaining varied across samples for all molecules, but only the extent of αvβ6 integrin immunostaining was associated with increased mortality. There was no association with the other markers measured.

Conclusion

Our data suggest high levels of αvβ6 integrin may identify a specific endotype of progressive fibrotic lung disease.

INTRODUCTION

Fibrotic Interstitial Lung Diseases, including Idiopathic pulmonary fibrosis (IPF) and fibrotic Non-Specific Interstitial Pneumonitis (NSIP) are chronic, progressive, diseases associated with serious morbidity and premature death. The median survival for IPF is approximately 3 years [1]. Currently 4500 new cases of IPF arise each year in the UK, and both the incidence and mortality have been rising for the last few decades [2]. NSIP is considered a distinct clinical entity, with a better prognosis compared with IPF, although many patients suffer from progressive disease [3]. Furthermore, there is a large group of patients with unclassifiable ILD with a similar progressive, but heterogenous, clinical course [4]. The heterogenous nature of progressive fibrotic interstitial lung diseases suggests there may be endotypes of disease both distinct, and common, across the different clinical phenotypes.

There is considerable interest in defining endotypes of progressive fibrotic interstitial lung disease to both understand disease progression and to facilitate stratified treatment design. Transcriptional profiling is able to distinguish patients with stable disease from those who have progressive disease [5] and acute exacerbations [6], although no difference in mortality was documented. Studies of biopsies from patients with IPF have assessed whether the fibroblastic foci correlate with reduced survival but the results have not been conclusive [7 8], and molecular phenotyping for TGFβ signalling molecules did not show any relationship to survival [9]. Serum and plasma biomarkers such as KL-6 [10], CXCL13 [11] CCL18 [12], MMP3 [13] and MMP-7 [14], have been shown to have prognostic value in IPF although how they reflect underlying disease pathogenesis, or whether they change in response to therapy, remains uncertain. Recently it has been hypothesised that there may be two distinct endotypes of disease, related to disrupted bronchiolisation and lymphoid aggregates, which may prove useful in developing interventional clinical trials [13].

IPF is thought to result from initial injury of alveolar epithelium leading to activation of lung fibroblasts, which subsequently synthesise excess matrix. The $\alpha v\beta 6$ integrin is an epithelial restricted molecule that has been implicated in numerous models of lung fibrosis [15-18], in patients with IPF [16 18], and targeted therapy is currently undergoing phase 2 evaluation in the USA (clinicaltrials.gov NCT01371305). Surfactant Protein C (SPC) is another epithelial specific protein that has been associated with IPF. SPC levels are increased in IPF bronchoalveolar lavage, [19], and genetic studies have implicated SPC gene mutations in both sporadic and familial IPF [20]. The emergence of a myofibroblast phenotype, characterised by αSMA expression, is a key event in the pathogenesis of fibrosis and may be responsible for the increased contractility in tissues undergoing fibrosis [21]. HGF is increased in response to tissue injury, and inverse correlations have been identified for HGF expression during the development and progression of fibrosis in several tissues including the lung [22]. Furthermore, lung fibroblasts isolated from IPF patients have

decreased HGF expression and activation relative to fibroblasts from control patients [23]. Tenascin C is increased in fibrosis, especially in Usual Interstitial Pneumonitis (UIP), a hallmark of IPF [24]. It is abundant in the fibroblast foci of active fibrosis and is also present in the basement membrane regions beneath the metaplastic epithelium lining honeycomb cysts [25].

We evaluated these molecules by immunohistochemical analysis of fibrotic ILD tissue, as well as quantifying fibroblastic foci, to determine whether specific epithelial, or mesenchymal, endotypes of progressive fibrotic lung disease exist to facilitate clinical trial stratification.

METHODS

Reagents

Xylene (X-0200-17) and 100% ethanol were purchased from Fisher UK. Hydrogen peroxide (30% H_2O_2), Methanol (95294-1L), 10mM Sodium citrate buffer, Horse serum (H0146-10ML), Goat serum (G-9023), Trizma® base, SIGMAFAST™ 3,3'-Diaminobenzidine tablets, Mayer's Haematoxylin and Eosin (MHS32) were purchased from Sigma-aldrich, Dorset, UK. Commercially available ABC kit was used from Vectastain Universal ABC Kit. The antibodies used were anti-αSMA mouse monoclonal (ab7817, clone 1A4, Abcam), Tenascin C mouse monoclonal (NCL-TENAS C, Novocastra), anti-c-met mouse monoclonal (Invitrogen, cat no 37-0100) and anti-pro surfactant protein c rabbit polyclonal (Millipore NG1850802) were used. The anti-αvβ6 integrin antibody (6.2A1, Biogen Idec) was generated as previously described [26]. The secondary antibodies used were biotinylated horse anti-mouse, biotinylated goat anti-mouse and biotinylated goat anti-rabbit (Vector Laboratories, Peterborough, UK).

Patient samples

Forty-three ILD lung sections were obtained via Video-Assisted Thoracoscopic Surgery (VATS) biopsy, which were formalin fixed and paraffin-embedded. The sections were obtained from W Wallace (University of Edinburgh). These patients were seen in outpatient clinic in Edinburgh from the period 1999-2009 and required biopsy for diagnostic purposes. All patients had a diagnosis of IPF or NSIP as per the international consensus criteria [27]. They were subsequently followed over a 10-year period to allow analysis of "all cause" mortality; unfortunately there are insufficient clinical data to determine the cause of death. The tissue samples were obtained after informed consent and local ethics approval (Code-South East Scotland SAHSC Bioresource 06/S1101/41).

Immunohistochemistry

Parallel lung tissue sections were cut and deparaffinised in xylene followed by rehydration in decreasing concentrations of ethanol and washed in Tris-buffered saline. Antigen retrieval, primary and secondary antibody for each marker has been listed in Supplementary Table 1 (appendix, web only file). The endogenous peroxidase activity was blocked using 3% H_2O_2 in methanol for 30 mins. The sections were washed twice with Tris-buffered saline and incubated with a buffer block of 5% goat or horse serum, for 20 min at room temperature (RT). Sections were incubated with primary antibody for 1 hour at RT, or overnight at 4°C. Subsequently, sections were washed with Tris-buffered saline and incubated for one hour with biotin-conjugated secondary antibody. Signal amplification was performed using the Vectastain standard ABC kit prior to visualisation with DAB for 10 min. Slides were counter stained in Mayer's hematoxylin (Raymond A Lamb Limited, East Sussex, UK). Secondary antibody only controls were used for all experiments.

Quantification Analysis

Each patients' sample was analysed for two epithelial markers ($\alpha v\beta 6$ integrin and SpC) and three mesenchymal markers (αSMA, HGF and tenascin C), therefore 43 samples were analysed for five immunomarkers. The interpretation of histological sections is an inherently subjective process based primarily on morphologic features. When used by an experienced operator, the scoring methods have been shown to have statistically significant correlation with clinical outcomes [28]. Automated analysis has been increasingly used in recent years to attempt to standardize quantification. In the absence of a clear consensus on the most accurate quantification method, two techniques incorporating both semi-quantitative and automated analysis were used.

Operator dependent, semi-quantitative analysis

Individual patient slides contained between one and five lung sections. Five high power fields (HPF) per tissue section (maximum of 25 per slide) were analysed. To normalise for varying numbers of regions of interest per slide the mean score per slide was calculated. For epithelial markers the degree of staining is expressed as the percentage of the epithelial surface in selected HPF. For the purpose of quantification it was assumed that all cells on the luminal surface were epithelial in origin. For the mesenchymal markers, the whole section was taken into account for analysis and the degree of staining is expressed as a percentage of the whole section. To avoid overlapping analysis of individual tissue sections, the slides were viewed in a structured manner from right to left, both when analysing each tissue section and each slide. The coding was done prior to analysis and is as follows:

0=Negative

1=0-≤30%

2=30-≤50%

3=50-≤70%

4=≥70%

Each slide was analysed by two independent investigators blinded to the outcome of the patient. Weighted Kappa analysis was done to measure agreement between two operators. In patients where there was a disagreement, an average of the two was done for analysis.

Automated image analysis

Automated image analysis using Nikon 90i microscope image analysis software (NIS Elements) was performed. A threshold using the RGB mode was selected to determine the pixels to be included in the section. The threshold limit was defined using reference points from within the image in positive control. For epithelial surfaces, a single point tool was used to define threshold, whilst a 3-point circle was used for the mesenchymal markers. Using the Threshold ND image tool, the thresholding was uniform for all frames. An algorithm to define a histological region of interest (ROI), to define signal localisation was used. The ROI was selected at random and was fixed in terms of area. Five HPF per section (up to maximum of 25 per slide) were taken for the purpose of analysis. The amount of stain is represented as percentage of the ROI area, with average of the five HPF as numerator and ROI area as denominator.

Fibroblastic Foci quantification

Fibroblastic foci quantification was performed as previously described [8]. The following criteria were used to identify fibroblastic foci based on previously published definitions [8 29]:

- Discrete sub-epithelial areas of spindle shaped myofibroblasts and fibroblasts
- Linear arrangement of cells parallel to luminal surface
- Pale staining matrix in contrast with darker parenchyma

Five fields, using x10 objective, per tissue section (maximum of 25 per slide) were analysed. Each slide was analysed by two independent observers and mean score of the two was used for analysis.

Statistics

The start date was the date of the biopsy. The end date was taken as the date of death or the last known date the patient was alive (15/11/2011). Patients were censored on the last known date that they were alive (15/11/2011). There is no gold standard for IHC analyses hence the groups were divided into quartiles for operator dependant and tertiles for automated analysis. Ranked analysis has been done and p-value for trend has been shown. Cox's Proportional Hazards Regression and Kaplan-Meier for survival analysis were carried out. The quantification was done blinded to the survival data. Spearman correlation was done between operator dependent and automated analysis. StataCorp. 2009. *Stata Statistical Software: Release 11*. College Station, TX: StataCorp LP was used.

RESULTS

Patient demographics

Basic demographic data are shown in Table 1. The median age of patients was 63 years with a slight predominance of males. 36 patients had UIP and five had NSIP.

Table 1: Demographics of 43 ILD patients undergoing VATS biopsy at Edinburgh, UK (1999-2009).

Age (median)	63 years (43-76years)
Sex (M:F)	24:19 (56% vs 44%)
Diagnosis (UIP:NSIP)	36:5 (#2 were unclassifiable fibrotic ILD)
Median survival after biopsy (N=43)	68 month (11 months-115months)

ILD patient biopsies stain positively for a range of immunomarkers

All sections stained positively for the five immunomarkers assessed when compared with secondary antibody controls (See figure 1 and figure 2). $\alpha v\beta 6$ integrin and pro-SpC stained only the epithelium (figure 1a and 1b), consistent with their known cellular distribution. αSMA stained the myofibroblast in the mesenchymal regions of the fibrotic lung parenchyma (figure 2a). HGF was expressed diffusely in the interstitium, excluding the epithelial surfaces (figure 2c). Tenascin C showed positive staining in the fibrotic interstitium and stromal fibres underlying the immune-negative epithelium (figure 2b).

Relationship between immunostaining and prognosis

Weighted kappa agreement between the two investigators recording semi-quantitative analysis was 81%. Furthermore, the semi-quantitative and automated analysis was strongly associated (Rs 0.83, $p<0.0001$). Of the five immunomarkers assessed only $\alpha v\beta 6$ integrin was found to be associated with prognosis, with high levels of immunostain being associated with a worse prognosis using both operator dependent and automated image analysis methodology (see table 2a & 2b). There was a four-fold increased risk of death (95%CI 0.87-20.23) in patients expressing highest levels of $\alpha v\beta 6$ integrin using the operator dependant analysis (p-trend: 0.0019), and two times increased risk of death (95%CI 0.75-5.7) in high expressers of $\alpha v\beta 6$ integrin using the automated analysis (table 2a). A worse median survival of 25months was seen in patients with highest expression of $\alpha v\beta 6$ integrin (see figure 2; log rank equality test, $p<0.0001$). Differences in survival are shown in the Kaplan-Meier curves (figure 3).

A similar trend was also seen with αSMA using the operator dependant analysis with high levels of immunostain being associated with shorter survival, however these results did not reach statistical significance by either methodology (Table 2a).

There was no association between levels of immunostain of HGF, Ten C and pro-SpC and prognosis by either methodology (see Table 2b). The automated image analysis for pro-SpC, using the RGB thresholding was giving a false high positivity thus only operator dependant results are shown. There was no difference in the results on excluding the unclassifiable biopsies.

Relationship between fibroblastic foci and prognosis

H&E staining was used to identify fibroblastic foci (Figure 4a). Weighted kappa agreement between the two investigators recording fibroblastic foci was 68%. Using the Cox proportional model, there was a 33% increased risk of death with increasing number of fibroblastic foci (HR 1.33, 95%CI 0.72-2.46), however this was not statistically significant (Figure 4b).

Table 2a: Adjusted Hazard's ratio and 95% confidence interval (CI) for relationship between quantitative immunostaining and death in 43 ILD patients, 1999-2009.

Biomarker	**Method**	**Group (n=number of patients)**	**Hazard ratio**	**95% CI**	**p value for trend**
αvβ6	**Semi-quantitative**	0-30%*(n=3)*	1		0.0019
		30-≤50% *(n=14)*	0.71	0.134-3.80	
		50-≤70% *(n=12)*	0.76	0.15-3.88	
		>70% *(n=14)*	4.19	0.87-20.23	
	Automated	Group 1*(n=15)* Low expression	1		0.25
		Group 2*(n=14)* Medium expression	0.98	0.32- 3.06	
		Group 3*(n=14)* High expression	2.07	0.75-5.7	
αSMA	**Semi-quantitative**	0-30% *(n=2)*	1		0.49
		30-≤50%*(n=8)*	0.99	0.10-9.74	
		50-≤70% *(n=12)*	4.54	0.49-41.93	
		>70% *(n=21)*	1.77	0.22-14.01	
	Automated	Group 1*(n=15)* Low expression	1		0.72
		Group 2*(n=14)* Medium expression	1.23	0.41-3.73	
		Group 3*(n=14)* High expression	1.54	0.52-4.54	

Adjusted for age and sex

Table 2b: Adjusted Hazard's ratio and 95% confidence interval (CI) for relationship between quantitative immunostaining and death in 43 ILD patients, 1999-2009.

Biomarker	**Method**	**Group (n=number of patients)**	**Hazard ratio**	**95% CI**	**p value for trend**
HGF	**Semi-quantitative**	0-30%*(n=1)*	1		0.94
		30-<50%*(n=12)*	0.63	0.074-5.30	
		50-<70%*(n=13)*	0.60	0.070-5.09	
		>70%*(n=17)*	0.71	0.087-5.75	
	Automated	Group 1*(n=15)* Low expression	1		0.41
		Group 2*(n=14)* Medium expression	1.70	0.65-4.48	
		Group 3*(n=14)* High expression	0.84	0.28-2.52	
SPC¶	**Semi-quantitative**	0-30%*(n=12)*	1		0.99
		30-≤50%*(n=14)*	1.17	0.37-3.70	
		50-≤70%*(n=9)*	1.25	0.32-4.78	
		>70%*(n=8)*	1	0.30-3.28	
Tenascin-C§	**Semi-quantitative**		1.16	0.36-1.97	0.58
	Automated	Group 1*(n=15)* Low expression	1		0.57
		Group 2*(n=14)* Medium expression	1.62	0.55-4.83	
		Group 3*(n=14)* High expression	1.76	0.55-5.62	

¶ Automated analysis was not possible for SPC due to a high degree of false positive staining.
§ Only the trend for semi-quantitative method is shown, as there were no deaths in Group 4. Adjusted for age and sex.

DISCUSSION

These data show that high levels of αvβ6 integrins are significantly associated with poor prognosis. There is also a trend towards worse prognosis with high levels of αSMA. However, there were no differences observed between samples with UIP or NSIP histology, although this study was not sufficiently powered to detect a difference between the two groups. Furthermore, there was no apparent relationship between the number of fibroblastic foci and mortality consistent with previous reports [8]. This is the first study to demonstrate a tissue immunomarker in ILD with a significant association with the prognosis. A notable observation is the median survival of patients with the highest expression of the αvβ6 integrin was only 25 months, which is comparable to the published survival data in IPF. This suggests that increased expression of the αvβ6 integrin may represent a distinct endotype of progressive fibrotic ILD and could be useful as a biomarker for disease progression, and stratification of therapy.

There have been a number of studies that have tried to characterise molecular endotypes in ILD [7 30-32]. The phenotype, which has been consistently shown to associate with poor survival on lung biopsy, is the presence of high levels of fibroblastic foci [7 9 32]. However, the assessment of fibroblastic foci relies on conventional histological techniques, and does not reflect precise molecular endotyping. We did not find a significant association between either high numbers of fibroblastic foci, or high levels of αSMA, and prognosis. These data are consistent with previous studies which have found only weak, non significant, correlations of active myofibroblasts with disease severity [31]. However, there was a trend towards a worse prognosis with higher levels of both fibroblastic foci and αSMA, which supports the notion that fibroblastic foci are an adverse feature in IPF biopsies but larger studies may be required to accurately quantify this effect.

The role of αvβ6 integrins in the pathogenesis of pulmonary fibrosis is well established and αvβ6 immunostaining is increased in IPF, compared with control lung tissue [16-18]. Furthermore, a humanized anti-αvβ6 monoclonal antibody, STX-100, is currently being evaluated in a Phase 2 study. However, the association of αvβ6 integrin levels with prognosis of fibrotic ILDs has not been previously assessed. However, endotyping by tissue biopsy is an invasive procedure carrying significant mortality and morbidity irrespective of the mode of biopsy [33 34]. The short term mortality post lung biopsy in IPF is higher compared with other interstitial diseases[35]. Furthermore, there is an inherent sample bias in the surgical biopsies due to the patchy nature of the disease, and assessment of a limited number of tissue sections within the whole lung biopsy. This is noted irrespective of the modality of the procedure undertaken to obtain the surgical lung biopsies (VATS, Open lung or transbronchial lung biopsies) [36]. An alternative to immunohistological assessment of lung biopsies could be through CT/SPECT imaging. This technique is highly sensitive and able to globally quantify αvβ6 integrin levels within the lung [37]. Therefore, molecular

imaging of the αvβ6 integrin may have advantages over immunohistochemistry as a prognostic biomarker in fibrotic ILDs.

The strength of the study is the robust quantification methodology used for the tissue biomarkers. This study shows that operator analysis is similar to automated analysis. At present, the automated analysis is an expensive modality and often a research tool, whereas chromogenic staining is a well-established technique requiring only a standard microscope. In parallel with advances in cancer management, recent NICE guidance in United Kingdom proposes a multidisciplinary team (MDT) approach to IPF, and emphasises the importance of the pathologist [38]. The importance of these members of the MDT will become even greater if molecular phenotyping of lung biopsies follow the trajectory observed in lung cancer. However, interobserver variation and pathologist kappa scores are moderate even in the most robust of studies [32 39], and the number of biopsies to be performed is likely to remain a small proportion of the total, due to the technical issues described previously. Therefore, exploring non-invasive strategies to define prognosis and response to therapy remains crucial.

The main limitation of the study lies in the small cohort size. Despite this the association of high levels of αvβ6 integrin immunostaining with poor outcome was significant. Furthermore, a trend suggesting that high levels of αSMA, and high numbers of fibroblastic foci, also lead to worse prognosis supported previous observations, and a larger cohort may aid confirmation. Whether a large prospective study defining the role of immunomarkers in IPF is a possibility, given the limitations of the diagnostic lung biopsy, in contrast with the strength of the diagnostic CT scan, remains to be seen. Another limitation of the study is the absence of a control population, however this study was designed to address the role of these molecules as prognostic, not diagnostic biomarkers. Conventional controls for IPF immunohistochemistry are areas of normal lung taken from lung cancer patients undergoing lung resection and clearly this would not be an appropriate control for a prognostic study and patients with truly normal lung do not undergo lung biopsy.

Despite the limitations of our study, the observations described are important because they highlight that molecular markers have potential to aid stratification and the determination of prognosis in IPF. Furthermore, given the importance of histopathology in the management of ILDs, it is vital that as much information can be gained from biopsies as possible, and molecular endotyping may increase their utility. Ultimately this study demonstrates that high levels of the epithelial restricted αvβ6 integrin may identify an endotype of progressive fibrotic ILD with a poor prognosis, which may be appropriate for therapeutic targeting.

Acknowledgements: South East Scotland SAHSC BioResource for access to the tissue. Alison John (University of Nottingham) for help with the Nikon image analysis software.

Funding: MRC MICA grant G0901226

Competing interests:

GJ: Consultancy fees received from GlaxoSmithKline, Intermune, Boehringer Ingelheim, MedImmune, Pulmatrix and Biogen Idec. Research contracts received from GlaxoSmithKline, Novartis and Biogen Idec. Lecture fees received from Boehringer Ingelheim, Intermune and MedImmune.

SMV and PHW: Employees and shareholders of Biogen Idec,Inc.,the developer of STX-100.

Contributorship

GS: Planning and conducting the study, analysis and writing the manuscript.

JP: Planning the experimental protocols, manuscript review.

PHW and SMV: Planning experimental Immunohistochemistry protocols and manuscript review.

WAW: Tissue sections were given by WAW, planning Immunohistochemistry analysis and manuscript review.

TMM: Statistical planning and reporting, reviewing the manuscript.

GJ: Original concept, manuscript reviewing and overall guarantor.

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Figure legends

Figure 1: Immunohistochemical staining in paraffin-embedded, formalin fixed surgical lung biopsies in 43 fibrotic ILD patients. Immunostaining shows high levels of Epithelial markers: αvβ6 integrin and pro-SpC compared with secondary antibody only control as comparator. Representative sections of IPF lung are shown with control for each immunomarker.
Immunostaining magnification= originalx20.
a) αvβ6 integrin staining in the alveolar epithelial cells.
b) pro-SpC immunostain in the cytoplasm of the epithelial cell layer in a patchy distribution.
c) αvβ6 integrin secondary antibody control
d) pro-SpC secondary antibody control

Figure 2: Immunohistochemical staining in paraffin-embedded, formalin fixed surgical lung biopsies in 43 fibrotic ILD patients. Immunostaining shows high levels of Mesenchymal markers markers: αsma, HGF and Ten C, compared with secondary antibody only control as comparator. Representative sections of IPF lung are shown with control for each immunomarker.
Immunostaining magnification= originalx20.
a) αsma immunostain within the fibroblastic foci, showing cluster of cells composed of myofibroblasts.
b) Ten C immunostain in the stromal fibres beneath the immune-negative epithelial layer.
c) HGF present diffusely in the interstitium along with negatively immune-stained alveolar epithelial cells, consistent with its known location.
d) Secondary antibody controls for a-c.

Figure 3: Kaplan-Meier survival for αvβ6 integrin immunomarker in 43 ILD patients with VATS biopsy. Survival graph of αvβ6 integrin expression, with the highest expressing Group 4 (bold line) vs Group 1 (dashed line), Group 2 (short dashed line) and Group 3 (dotted line). Median survival of Group 4 vs. Group 1-3 was 25 months and 92 months respectively (p=0.0019).

Figure 4: Assessment of fibroblastic foci (FF) in paraffin-embedded, formalin fixed surgical lung biopsies in 43 fibrotic ILD patients.
a) Section of lung tissue illustrating FF (highlighted by arrows) using 10x objective. Scale bar shows 100μm.
b) Kaplan-Meier survival of fibroblastic foci in 43 ILD patients following VATS biopsy. Group 1 (<1.3 FF/section), Group 2 (1.3<3 FF/section) and Group 3 (3<7 FF/section).